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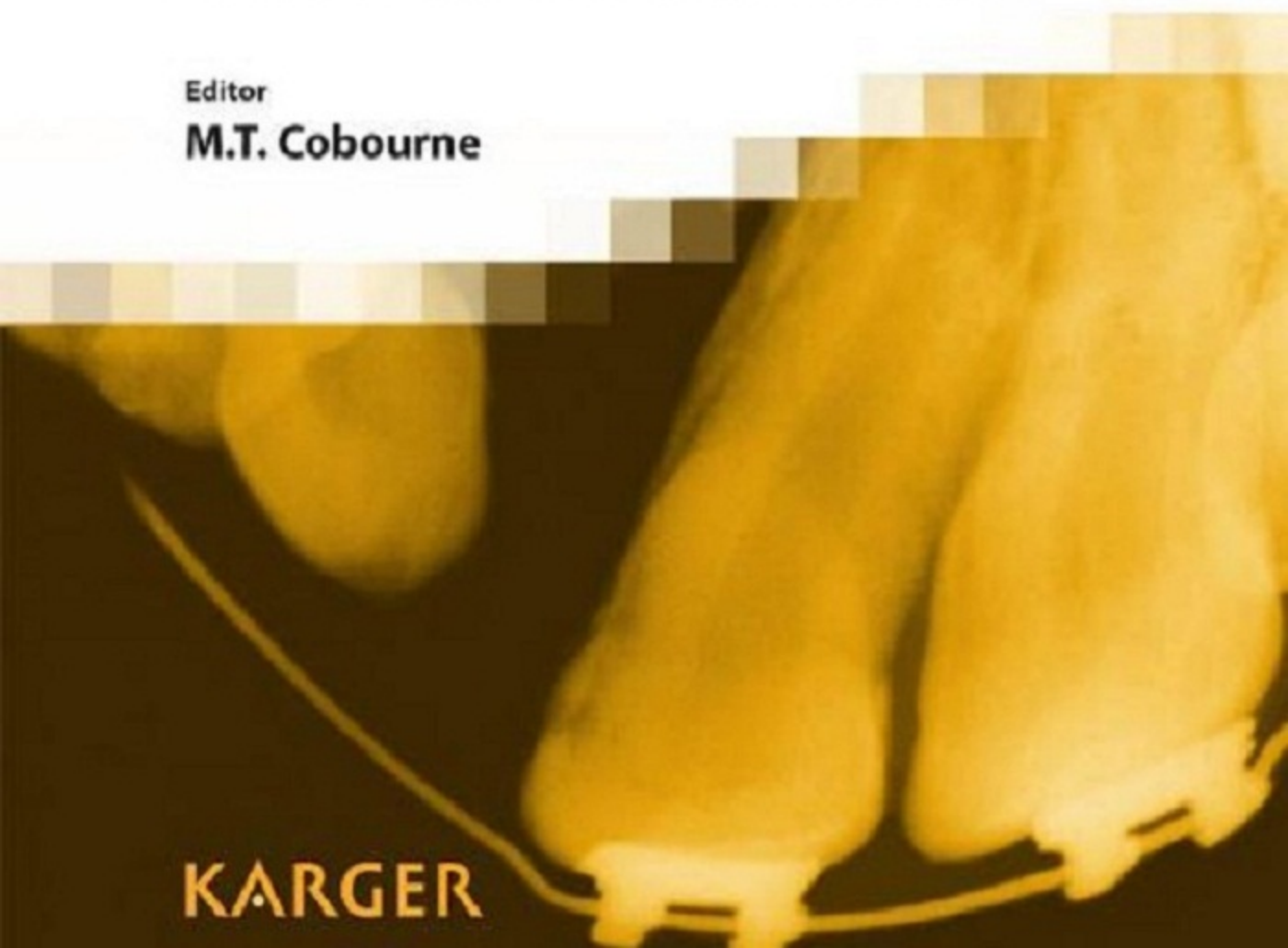
Cleft Lip and Palate

Epidemiology, Aetiology and Treatment

Editor

M.T. Cobourne

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Cleft Lip and Palate

Frontiers of Oral Biology

Vol. 16

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Cleft Lip and Palate

Epidemiology, Aetiology and Treatment

Volume Editor

Martyn T. Cobourne London

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Foreword

There is some truth in the old adage that ‘an expert is someone who knows more and more about less and less’. Craniofacial biology was simpler when I was a Glasgow dental student in the 1960s. Knowledge of normal and abnormal orofacial development was mainly derived from the histological dissection of animal and human embryos, accompanied by speculation on the growth mechanisms responsible. Understanding of those mechanisms has of course been transformed in the ensuing half century with the advent of new laboratory technologies, animal models, genome-wide sequencing, and so forth.

On the other hand, evidence around clinical care, with some exceptions, has hardly changed. Indeed many of the historical disputes regarding best surgical techniques and the value or otherwise of adjunctive procedures such as presurgical orthopaedics (an even earlier Glasgow export) continue to the present day. But happily, there are signs that we are beginning to dig ourselves out of this mire, with the emergence of well-structured multicentre comparisons and even well-powered randomised trials.

One of the high points of my time in clinical cleft research was participation in a European Commission project known as Eurocran (2000–2005), which forged a research partnership of clinicians, geneticists, laboratory scientists, and epidemiologists, and which continues in various forms today. Almost by osmosis, we began to grasp the importance of each other’s research in

the thick mix of initiatives required to advance understanding, care, and prevention.

Accordingly, this book fills a significant gap in the literature, providing an update on the state of the science concerning the aetiology and mechanisms responsible for clefts of the lip and palate, together with an overview of contemporary clinical management.

The first section provides a thorough international review of the epidemiology of orofacial clefting, affirming that non-syndromic, non-chromosomal clefting has a polygenic multifactorial aetiology that exhibits geographic and racial variations. In particular, variations emerging on the basis of sub-phenotype, gender and exposure to environmental factors in themselves raise important research questions about cause and ultimate prevention. Complementing this is a review of the considerable progress in gene identification over the last two decades, alongside the influence of environmental factors. Future advances in these areas will require collaboration between clinicians and scientists.

The second section explores the coordination of different tissues and signalling pathways necessary for palatogenesis, via cutting-edge research involving mouse models. A helpful chapter providing an account of the stages of palatogenesis and the critical cellular and molecular mechanisms that accompany each step provides a clear framework for assimilating the more detailed accounts of individual growth factors that follow.

The last section covers the principal elements in clinical care. Surgical anatomy, primary surgery and major secondary procedures are covered, and the uncertainty in selecting protocols of primary surgery in particular is acknowledged. Orthodontics and speech therapy are presented in a pragmatic way with emphasis on teamwork and a choice of interventions designed to optimise outcome while minimising burden. A full account of alveolar bone grafting, possibly the most important addition to cleft care in recent decades,

is included. Finally, the importance of monitoring outcomes and collaborative multicentre working in research are stressed.

Bill Shaw

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Preface

Clefts affecting the lip and palate are a relatively common group of developmental anomalies that occur in many human populations. They are seen in isolation or in combination with more widespread developmental disease, and can contribute towards significant morbidity for affected individuals, particularly in their formative years, which inevitably has an impact throughout their lifetime. These conditions are the focus of research for scientists within many different specialties, and this is reflected in the current volume which is divided into three broad sections describing current concepts in the epidemiology, molecular biology and contemporary management of cleft lip and palate.

In the first section, the etiological basis of human cleft lip and palate is discussed in the broadest sense, with focus on the epidemiology and underlying genetic and environmental contributions to this condition. In the second section, the developmental biology of early lip and palate development is discussed. There is an extensive discussion of the predominant developmental model used to investigate facial development, the mouse, whilst the principle molecular signalling pathways involved in lip and palate development are also described (Hedgehog, Bone Morphogenetic Protein, Fibroblast Growth Factor and WNT), with focus on their role in the aetiology of oro-facial clefting. Finally, in the third section, current concepts and controversies in the management of cleft lip and palate are discussed, including the assessment of

treatment outcome, interventions for primary surgical correction, orthodontic treatment, alveolar bone grafting and the management of speech and language development. In the final chapter, the use of molecular tools in the prevention and treatment of cleft lip and palate is discussed, highlighting how translational research might offer possibilities to harness knowledge of the underlying developmental biology of lip and palate formation to prevent or more effectively treat cleft lip and palate in affected human populations. Once again, these experiments are being conducted primarily in the mouse, but they offer exciting possibilities for future strategies aimed at combating this common developmental anomaly.

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Epidemiology of Oral Clefts 2012: An International Perspective

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Abstract

Classical descriptive epidemiology in the field of cleft lip and palate aims to quantify the problem, and in the higher income countries it is possible to do this with varying degrees of accuracy. This is not however possible in every country in the world, and epidemiology should seek to identify these data gaps with a view to improvement in the situation. Epidemiology must also be investigative and look for trends, associations and inter-population differences, with the aim of supporting aetiological research and advancing the translational agenda. This chapter is set out in three parts and seeks to address all three of the above areas. Birth defects in general and orofacial clefting in particular remain a relatively common and significant problem for not only the individual patients born with these defects in terms of death or disability, but also for their families and for society in general in terms of burden of care and health inequality. In high-income countries, despite very significant advances in treatment, problems in access to care and evidence base for cleft care still exist whereas in the developing world the consequences are lack of access to care and lack of infrastructure to help with quantification of the problem and consequently the ability to address it. The major questions in contemporary cleft lip and palate research surround ways of improving the evidence base for the treatment interventions used to optimise quality of care, and the ultimate scientific and humanitarian objective is primary prevention of those dis-

eases and disorders that are preventable. Descriptive epidemiology underpins research enquiry in both of these major areas.

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Part 1: Descriptive Epidemiology for Orofacial Clefts

In 2002, a comprehensive overview of orofacial cleft (OFC) epidemiology up to the end of the 20th century which included a systematic literature search and a review of the major international registries including EUROCAT, ICBDMs (incorporating ECLAMC figures) and NBDP was published [1]. The available data indicates that the overall figure for OFC prevalence is approximately 1 in 700 live births with considerable ethnic and geographical variation. The following summarises the main findings.

Geographical Variation in Prevalence

International data from 57 registries for the period 1993–1998 suggests about a seven-fold variation in the prevalence at birth of CL(P), ranging from 3.4 to 22.9 per 10,000 births, and even more pronounced variation for CP, with prevalences ranging from 1.3 to 25.3 per 10,000 births [2]. Table 1 shows average prevalence by Global Burden of Disease (GBD) region calculated using the data of Mossey and Little. EUROCAT registries data

Table 1. Estimated birth prevalence of OFCs by GBD region: ranked in descending order of prevalence (chromosomal excluded)

GBD region	Total OFCs/1,000 (non-chr)	CP/1,000 (non-chr)	CL(P)/1,000 (non-chr)	CP, % of total
Latin America, Southern	2.39	0.72	1.67	30
Latin America, Tropical	2.39	0.72	1.67	30
Australasia	2.01	1.02	0.98	51
North America, High Income	2.00	0.83	1.17	41
Oceania	1.85	1.13	0.72	61
Europe, Western	1.66	0.59	1.07	35
Asia Pacific, High Income	1.65	0.64	1.00	39
Asia, South	1.60	0.30	1.30	19
Latin America, Central	1.54	0.39	1.15	25
Europe, Central	1.45	0.67	0.77	47
Asia Southeast	1.36	0.28	1.08	20
Latin America, Andean	1.29	0.17	1.12	13
Asia, East	1.28	0.27	1.01	21
Europe, Eastern	1.22	0.59	0.63	49
Asia, Central	1.19	0.62	0.57	52
Middle East	1.02	0.30	0.72	29
Caribbean	0.93	0.31	0.62	34
Sub-Saharan Africa, Central	0.54	0.04	0.51	7
Sub-Saharan Africa, West	0.54	0.08	0.46	15
Sub-Saharan Africa, Southern	0.45	0.15	0.30	33
North Africa	0.44	0.15	0.29	35
Sub-Saharan Africa, East	0.38	0.12	0.27	31
World	1.25	0.31	0.94	25

(1995–2007) aggregated by country reveal that there is a greater than two-fold difference in prevalence of non-syndromic, non-chromosomal orofacial clefts in different parts of Europe, ranging from around 2/1,000 in Northern Europe to 1/1,000 in Italy. The mean is 1.36/1,000 (fig. 1). It is felt that within Europe the differences identified between countries are real and not due to variation in case ascertainment and criteria for collection because of the consistency in the methodology and multiple sources of ascertainment employed in EUROCAT. Higher rates of CL(P) were observed in parts of Latin America and Asia (China, Japan) and lower rates in Israel, South Africa and Southern Europe. Higher rates of CP were reported for Canada and parts of Northern Europe, and lower rates from parts of Latin America and South Africa.

Ethnic Origin, Migration and Population Admixture Studies

Comparisons between ethnic groups within the USA [3, 4] and the UK [5], and studying immigrants to the USA from Japan and China [3, 6], indicates that migrant groups have rates of CL(P) closer to the area from which they originated than those in the area into which they have moved. Whilst there are only a few studies carried out in Africa to examine cleft prevalence, these suggest a low prevalence of both CP and CL/P. African-Americans have lower rates for both CP and CLP than whites in the USA and a study in Birmingham (UK) also showed that those originating from the Caribbean have low rates of oral clefting [5]. Studies in North America also reveal high rates of CL/P in Japanese and Chinese [3, 7].

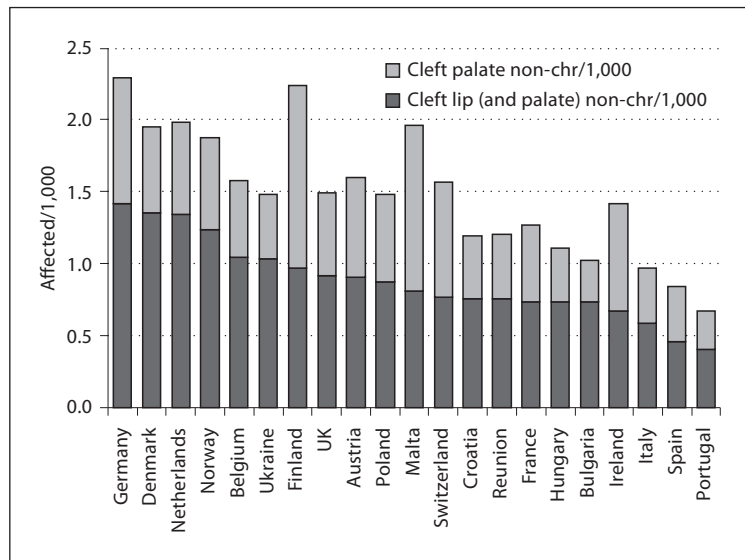


Fig. 1. Prevalence of non-syndromic/non-chromosomal orofacial clefts (CL(P) and CP) in Europe.

Relative Proportions of Different Cleft Types

European and US studies on non-syndromic cleft prevalence in general suggest that unilateral CLP is the most frequent single type of cleft accounting for about 30–35%. Isolated CL and CP each account for between 20 and 25% and BCLP is the rarest (about 10%), with submucous and other clefts accounting for the rest [8]. Within CLP, approximately 80% are unilateral and 20% are bilateral. Overall, 15% of all oral clefts are syndromic (12% of CL/P and 25% of CP) with over 300 syndromes recognised. Of the remaining 85% of individuals with OFC, 50% have other less well-defined anomalies [9].

Dysmorphological Severity of Cleft and Additional Malformations

The general trend among CL/P is that in those regions of the world where cleft prevalence is highest, the ratio of CLP to CL is highest, and in regions of lowest cleft prevalence, the proportion of the more severe forms of clefting is correspondingly low. This finding, first observed by Mossey and Little [1] reviewing international

data, is confirmed in the IPDTC study [10] and is in agreement with multifactorial model which would predict that the higher the overall CL(P) prevalence, the greater the genetic liability within that particular gene pool, and therefore the more CLP as opposed to CL. This model would also predict a greater proportion of bilateral as opposed to unilateral clefts in high prevalence populations, but such information was often not available.

Dysmorphological severity of CL(P) is associated with the severity of the phenotype in terms of other malformations, with a striking trend. Hagberg et al. [8] reported an increase in additional malformations in the bilateral subgroup of CL(P). Cases with bilateral CLP should be investigated for associated malformations since there is a three-fold increased risk of having another serious malformation, compared with unilateral CL. Co-morbidity is an aspect of cleft lip and palate epidemiology that is not well documented and may well have implications for aetiology [10–12].

This and more recent twin data on OFC from Denmark [13] provide compelling yet

indirect support for the multifactorial threshold model (MFT) and supports the notion of non-syndromic, non-chromosomal clefts being a threshold characteristic with genetic predisposition.

Unilateral Clefts and Laterality

Cleft lip only (CL) tends to be unilateral (around 90%) and approximately two-thirds occur on the left side regardless of sex, ethnic group and severity of defect [14–18]. In the IPDTC study [10], the proportion of bilateral cases was 10.3% (CI 95% 8.5–12.3) among CL and 30.2% (CI 95% 28.3–32.1) among CLP, with little variability among the registries or areas. In these registries the distribution by side (right or left) was given in 1,264 out of a total 2,506 unilateral cases. The proportion of right side was 36.9% (CI 95% 32.7–41.4) for CL and in 41.1% (CI 95% 37.6–44.7) for CLP. These proportions were very homogeneous among the registries and areas. No convincing explanation for these differences has been advanced but a proposed explanation is that blood vessels, supplying the right side of the fetal head, leave the aortic arch closer to the heart, and are perhaps better perfused by blood than those going to the left side [19].

Sex Distribution

Among the accepted epidemiological differences between CL/P and isolated CP is the now well-accepted male predilection to CL/P and female tendency towards CP, and the sex ratio varies with severity of the cleft [1], presence of additional malformations, the number of affected siblings in a family, ethnic origin and possibly paternal age [20]. In white populations the sex ratio for CL(P) is about 2:1 [1]. In Japanese populations there is a significant male excess in the CLP group but not in the cleft lip only group [21]. In white populations, the male excess in the CL(P) group becomes more apparent with increasing severity of cleft and less apparent when more than one sibling is affected in the family [14, 22]. By contrast,

the male predominance in CL(P) is attenuated when the infant has malformations of other systems [10]. No generally accepted explanation for these gender differences exists, although sex differences in the timing of critical developmental stages in craniofacial development are thought to have an as yet undefined role in their aetiology [23].

Associated Anomalies, Stillbirths and Termination of Pregnancy

There is considerable variation between studies in the proportion of cases with additional anomalies, but in general this appears to be more frequent for CP than CL(P) [1, 24]. It is possible that the presence of an anomaly of another system stimulates detailed examination leading to the detection of a mild CP that might not be reported had it occurred in isolation. In a study of almost 4,000 cases of CP in Europe, 55% occurred as isolated, 18% in association with other anomalies, and 27% as part of recognised syndromes [25]. For CL(P), a recent report of over 5,000 cases, 71% were isolated and 29% in association with other anomalies [25]. In combined data from European registries over the period 1995–1999, 3.5% of cases of CL(P) were still-born, and 9.4% from terminated pregnancies, and the corresponding proportions for CP were 2.4 and 8.1% [26], with most terminations where an OFC was part of the phenotype being for more severe associated anomalies. In a review of incidence of clefts between various racial or ethnic groups, Vanderas [27] concluded that the risk of developing clefts in stillbirths and abortions is approximately three times as frequent as in live births and also that clefts with associated malformations have a different epidemiological distribution than non-syndromic clefts (without associated malformations). He therefore suggested that incidence of clefts should be reported separately for live births, stillbirths, abortions and isolated separately from clefts with associated malformations and syndromes.

Clefts and Associated Malformations

From a global perspective there is variation in OFC with associated defects from as low as 21% reported by Milerad et al. [28], 29% reported by Shafi et al. [29], and by Calzolari et al. [25], and 31% by Rittler et al. [12], but Shaw et al. [30] reported a high prevalence of associated anomalies (59.8% for CL(P) and 71.1% for CP). All these studies show methodological differences, not least the definition of associated anomalies that hinder a reliable comparison, and inclusion of very minor defects contributes to the Shaw et al. [30] figure whereas in other studies a cleft was defined as associated only when major structural defects were present.

With regard to cleft type, CP has the highest prevalence of associated defects, followed by CLP and CL the lowest, as reported by Fraser and Calnan [15], Stoll et al. [31], Rawashdeh and Jawdat Abu-Hawas [32], and Rittler et al. [12], among others. A good representative example is a North Eastern France study which reported the rate of associated malformations as 46.7% in CP, 36.8% in CLP and 13.6% in CL [33], while Milerad et al. [28] reported the highest prevalence for CLP, followed by CP and CL.

Some studies also subdivide CL(P) into unilateral and bilateral groups when examining additional malformations and report an increase in additional malformations in the bilateral subgroup [8]. All studies that analysed CLP and CL separately [7, 24, 28, 31, 34] found the lowest prevalence of associated defects for isolated CL. Congenital heart disorders, limb and vertebral column anomalies were the defects that most often coexisted with clefts, both CP and CLP [12, 28, 29, 32, 34]. It is not clear whether the frequent association between congenital heart disorders and any other defect including clefts is genetically determined or coincidental and non-specific.

Updates in Global Epidemiology

Since 2002 there have been a number of significant international efforts through more systematic

registry-based systems to record the prevalence of orofacial clefts. A study conducted by EUROCAT [25] examined 6 million births in 23 EUROCAT registries in 14 European countries and identified 5,449 cases of CL(P) between 1980 and 2000. The overall prevalence of CL(P) was 9.1 per 10,000 with 70.8% isolated and 29.2% associated with either multiple congenital anomalies, chromosomal defects or recognised syndromes. Data from the IPDTC [10] examined birth prevalence data for CL(P) from 54 registries in 30 countries between 2000 and 2005 with a denominator of more than 7.5 million births. A very similar overall prevalence of 9.92 per 10,000 births was recorded, 76.8% of these being isolated and 23.2% either due to malformations in other systems (15.9%) or as part of a recognised syndrome (7.3%). For CP, the proportions of isolated, recognised syndromes and associated with other birth defects, i.e. multiple malformed infants, were 54.8, 27.2 and 18% respectively.

Data Deficiencies

Detailed descriptive epidemiology is confined to those parts of the world where infrastructure and resources allow, with substantial deficiencies in data from areas such as India, Sub-Saharan Africa and the Middle Eastern countries. This review of global epidemiology will therefore serve to reinforce the figures in those parts of the world where data are available and attempt to cast light on the areas where there are perceived problems with these data in terms of both availability and quality.

The validity and comparability of published prevalence data are affected by numerous factors, including: (a) the source population of births considered; (b) time period; (c) method of ascertainment; (d) inclusion/exclusion criteria; (e) clinical classification; and (f) sampling fluctuation. The developing world initiatives described in Part 2 of this chapter illustrate the problems that still remain with descriptive epidemiology in some parts of the world.

Contemporary Epidemiology

Contemporary study into the epidemiology of cleft lip and palate needs also to consider a range of other variables that are of interest such as: (a) cleft sub-phenotypes beyond the broad classification of CL(P) and CP; (b) microforms/genetic predisposition; (c) termination of pregnancy and stillbirths; (d) variation of cleft types with severity; (e) variation with latitude and longitude; (f) effect of folic acid on cleft severity, and (g) mortality and morbidity issues in OFC. These aspects are dealt with in Part 2 below.

Part 2: Descriptive Epidemiology from Selected Parts of the World Which Do Not Have Registries

Most of the data from which OFC epidemiology is derived are from the better developed, higher income countries where birth defects surveillance systems exist. Such infrastructure does not exist in many parts of the developing world, three of which are described below:

Sub-Saharan Africa

Prevalence of Orofacial Clefts

In 2002, Mossey and Little [1] reported on the relatively low birth prevalence in Sub-Saharan Africa, but the explanation for this remains ambiguous. It has been suggested that a high infant mortality rate, including unreported infanticide in children with lip or palatal clefts, could account for the low incidence and birth prevalence rates [35, 36]. Some studies reported higher rates for cleft lip and palate in Africa [37–41] compared to others that reported lower rates [35, 42] (see table 3), and studies from South Africa, where there is birth defects registration, reported low prevalence rates (0.3/1,000, 0.33/1,000) for the same geographical area [42, 43]. Although some of the studies with reported high rates included live birth and/or stillbirths in the base population, the observed population was small and with

a single source of ascertainment (table 2). All of these studies were hospital-based, which is not a true representation of the general population [44].

The study from Tunisia had a mixture of Arab and African populations which may be a reason for the higher rate reported from the study [41]. Also American ethnicity data, collected with the same method in a high-income setting, is very relevant for prevalence in Africa. Vanderas [27] reported that the incidence for all clefts combined in blacks in various US studies ranged from 0.18 to 1.67 per 1,000 births, and in one of these, the Myrianthopoulos and Chung [45] study of all clefts in 53,394 births they found 2.69/1,000 in whites and 1.67/1,000 in ‘negroes’. While these data still illustrate a relatively low prevalence for OFC in African-Americans, it is relevant to observe that the birth prevalence figures in a high-income setting where infant mortality is relatively low and ascertainment is presumably better are somewhat higher than those in Africa.

The report of more CL(P) in males and more CP in females from the African studies are consistent with the literature [46]. However the report of CLP as the subgroup with the highest associated congenital anomalies from the review is contrary to the literature which generally reports CP to have the highest associated congenital anomalies [11, 47].

The wide range in the confidence intervals in most of the studies (table 2) suggests that the precision is low and with hospital-based studies with poorly developed recording systems, there is reason to believe that (a) the ascertainment of cases is poor and (b) there is selection bias. For example, studies by Simpkins and Lowe [38] and Khan [37] did not provide any data for CP in the population.

India

In many parts of India the parents of a child born with a cleft have no access to counselling

Table 2. Prevalence at birth of clefts by type in small-scale, hospital/clinic-based epidemiological studies in Sub-Saharan African countries by country, population, number of clefts and prevalence

Study	Country	Denominator	Birth outcomes	Rates (95% CI)	Sample sizes			
					CL	CP	CL/P	all clefts
Simpkiss and Lowe 1961 [38]	Uganda	2,068	live births	1.45 (0.76–2.14)	2		1	3
Khan 1965 [37]	Kenya	3,016	live births	1.65 (1.08–2.22)	3		2	5
Gupta 1969 [39]	Nigeria	4,066	live births	0.95 (0.46–1.44)	1	1	2	4
Robinson and Shepherd 1970 [40]	Uganda	67,143 ¹	live births	0.75 (0.62–0.88)	19	6	22	47
Iregbulem 1982 [35]	Nigeria	21,624	live births	0.30 (0.12–0.48)	4	1	3	8
Kromberg et al. 1982 [42]	South Africa	29,633	live births and stillbirths	0.30 (0.13–0.47)	2 (1)	3	4 (1)	9 ²
Morrison et al. 1985 [43]	South Africa	9,377	live births	0.33 (0.25–0.41)	3	0	0	3
Khrouf et al. 1986 [41]	Tunisia	10,000	live births and stillbirths	1.50 (1.18–1.82)	6 (1)	5 (2)	4 (1)	15 ²
Ogle 1993 [36]	Zaire	56,637	live births	0.46 (0.32–0.60)	13	1	12	26
Msamati et al. 2000	Malawi	25,562	live births	0.67 (0.65–0.69)	1	16		17
Sulaiman et al. 2005	Sudan	15,890	live births	0.90 (0.64–1.16)	2	4	7 (1)	13

¹ Estimated size of population served by the hospital.

² Clefts with associated anomalies. Associated anomalies are shown in parentheses.

on the care and treatment of their children. Cleft lip and palate may be perceived to be a life-threatening abnormality in the absence of knowledge that clefts can be surgically repaired. Many infants with OFC die of malnutrition or

infection before they can access medical or surgical services. Birth defects surveillance systems are generally poor, but three multicentre studies in India have provided an almost similar frequency of CFAs.

Remarkable features of the population prevalence of OFC in India are (a) the striking discrepancies between urban hospital-based and rural population-based studies and (b) the low rates of isolated CP. Hospital-based CL(P) prevalence ranges from 0.93 to 1.3 per 1,000 births, and for CP the range is from 0.12 to 0.23 per 1,000 births. Based on these figures, the number of infants born with OFC in India is approximately 28,600 per year. However a thoroughly conducted population-based study of visible birth defects in 0- to 15-year-olds in the state of Tamil Nadu involving a population of 47.2 million people revealed a prevalence of OFC in the population of only 0.5 per 1,000 (0.2, 0.14 and 0.16 per 1,000 for CL, CLP and CP respectively) [Sridar, pers. commun., 2008]. This could be explained by the suspected high rates of infant mortality in those born with clefts and this is discussed in Part 3 of this chapter. In India, the first public health measure prior to setting up the interdisciplinary teams would be to have a more precise estimate of the prevalence and distribution of OFC in the Indian population, and there would be very significant advantages to making cleft lip and cleft palate notifiable diseases.

Saudi Arabia

While Mossey and Little [1] reported on data available at the time, there remains a dearth of information on the prevalence of OFC in parts of the Middle East, including Saudi Arabia – an example of a high-income country with underdeveloped birth defects surveillance. In Saudi Arabia the birth prevalence varies greatly from 0.23 in Riyadh to 2.19 per 1,000 live births in Al-Qaseem [48–50]. The mean prevalence of OFC for all the studies was 1.25 per 1,000 live births which is close to the global prevalence. The prevalence for each study is shown in table 3. Birth prevalence of different cleft types, laterality and sex ratios were all generally consistent with the literature.

The reported prevalence of consanguinity in families with OFC in Saudi Arabia and its neighbouring states range from 6.7 [49] to 83% [51] with

a high prevalence of first-degree cousin marriages [48]. The prevalence of a positive family history for OFC was between 23 [52] and 28% [48]. The prevalence of associated anomalies ranged from 12 [50] to 58.4% [52]. While more data are emerging, so too is evidence of major discrepancies between studies, due at least in part to inconsistent methodology and missing data on important issues such as family history and consanguinity [53].

Ascertainment

The relatively low proportion of CP in those countries which have poor birth defects surveillance raises questions concerning relative ascertainment of CL(P) and isolated CP. It is suspected that the rate of ascertainment for cleft lip with or without cleft palate will be reasonably high, but isolated cleft palate can go undetected at birth, and some, e.g. submucous cleft palate, can remain undetected until a problem presents later, for example a speech and language problem. Incomplete ascertainment of clefts has been noted in a recent Nigerian study [44] and is also suggested by Smile Train data from Ethiopia. This raises the possibility that under-ascertainment of CP contributes to the reported low prevalences in Africa and India.

Part 3: Other Characteristics of OFC Epidemiology

Sub-Phenotypes

As the information on genetic aetiology continues to be more refined it will be important to differentiate between CP and CL(P) sub-phenotypes, e.g. complete/incomplete and soft palate or hard palate clefts (CP) and to attempt genotype/phenotype correlation. There is increasing evidence for distinctive differences, both epidemiological and genetic, between CL and CLP. The different patterns of defects associated with CL and CLP, indicating different underlying mechanisms, suggest that CL

and CLP reflect more than just variable degrees of severity, and that distinct pathways might be involved [11]. Harville et al. [24] in a Norwegian study observed that 17% of infants with CLP had another defect compared with 9% of those with CL only. For males, the risk was greater for CLP than for CL ($p < 0.001$).

A recent UK study examined the extent of cleft lip in CL(P), and found that complete cleft of the lip in CLP patients was found to occur in 90% of males and 85% of females. In isolated cleft lip (CL) patients however, complete cleft of the lip occurred in the minority (39% of females and 25% of males) and the ratio of complete to incomplete CL was greater for females ($p < 0.0003$) [Carroll, unpubl. data, 2011]. The prevalence of a Simonart's band in patients with CLP ranges from 21.9 to 31.2% [54–58]. In the last of these studies the prevalence was higher BCLP than in UCLP and higher for the left side in UCLP and for the right side in UCL. The prevalence of Simonart's band in clefts of the lip and alveolus was much higher than in complete clefts of the primary and secondary palate (CLP). These descriptive epidemiological findings suggest aetiological differences.

Further evidence of genetic distinction comes from the Norwegian study [24] which found that the risk of CL only, but not of CLP, was increased for twins, and Rahimov et al. [59] reported that while CLP is strongly associated with SNPs in interferon regulatory factor 6 (*IRF6*), a study identified a common SNP (rs642961, G→A) in a novel *IRF6* enhancer allele was significantly over-transmitted in families with CL but not CLP.

Microforms

Orofacial clefting microforms: Genetic predisposition to orofacial clefts would be an extremely useful tool in the prediction of risk and therefore in genetic counselling and the prevalence of cleft microforms amongst the families of a proband born with a cleft of the lip or palate would therefore be important information.

The MFT hypothesis in the explanation of cleft prevalence: The MFT model can be used in polygenic multifactorial disorders to predict liability on the basis of cleft type, severity, sex and number of affected relatives and their genetic closeness to the proband. When applied to orofacial clefts, the MFT model predicts higher prevalence of the less severe types of cleft and males, being closer to the threshold for succumbing to CL(P) and females closer to the threshold for CP. In addition, the low MZ twin concordance rates also support a polygenic multifactorial aetiology with genetic heterogeneity and a significant environmental component.

Termination of Pregnancy and Stillbirths

In the IPDTC study [10], 13 of the 54 registries did not register ToPs and this may have influenced overall prevalence but the proportion of ToPs for CL(P) based on the informative registries in Canada, the USA and Eastern Europe was less than 5%, similar to what Forrester et al. [60] and Yazdy et al. [61] have surmised for isolated OFC in the USA. EUROCAT data [25] showed a termination rate of around 10% for pregnancies following prenatal diagnosis, was highest among MMC and syndromes and a close relationship was observed between the proportion terminated and the proportion with associated chromosomal abnormality. The highest proportions of ToPs were registered in the British Isles (14.3%), South-Mediterranean Europe (13.5%) and Western Europe (11.7%). ToPs were registered mainly 8.3% (184/2,228) among CLP and fewer 5.7% (73/1,283) among CL. In isolated cases, ToPs were rare 1.5% (33/2,228) among CLP and 0.9% (11/1,283) among CL. Some fetal deaths are reported where the fetus has OFC. It seems probable that most fetal deaths are due to other associated abnormalities but precise data is not readily available on this point at present. In EUROCAT the reported stillbirth rate was 1.8% for total orofacial clefts (isolated and associated) [26].

Table 3. Prevalence of OFC, and study characteristics for studies done in Saudi Arabia and surrounding Middle East countries

Reference	Site	Duration	Study design	Population examined
Kumar et al. 1991 [49]	KKUH ¹ , Riyadh, SA	1982– 1988	retrospective	20,045 live births/6 years in KKUH
Al-Johar et al. 2008 [48]	KFSHRC ² , Riyadh, SA	1999– 2008	retrospective	300,000 births/year in SA 80,000 births/year in Riyadh
Borkar 1993 [50]	KFSH ³ , Al-Qaseem, SA	1989–1992	1 year retrospective +3 years prospective	62,557 live births/4 years in Al-Qaseem from MOH birth registry statistics
Al-Talabani et al. 1998	Corniche Hospital Abu Dhabi, UAE	1992–1995	prospective	24,233 live births and stillbirths/3 years at Corniche hospital
Rajab and Thomas 2001 [52]	Khoula Hospital and all maternity sections in Oman	1989–1995	retrospective	375,000 births in all 2ry and 3ry level hospitals in Oman/7 years (80% of total population)
Patel 2009	10 health institution	2003–2005	retrospective	10,311
Al-Omary et al. 2004	KHMC ⁴ and Al-Bashir Hospital, Amman, Jordan	1991–2001	retrospective	1,548,106 births/11 years in Jordan
Aqrabawi 2008 [51]	KHMC ⁴ Amman, Jordan	Jan. 2000– Jan. 2005	prospective	25,440 live births/5 years

¹ King Khalid University Hospital.

² King Faisal Specialised Hospital and Research Centre.

³ King Fahad Specialised Hospital.

⁴ King Hussein Royal Medical Centre.

Prevalence, Type of Cleft and Dysmorphological Severity of Phenotype

Mossey and Little [1] observed an association between CL(P) prevalence and higher CLP:CL ratio. An extrapolation of this would be for the most severe type of clefts (bilateral CLP) being related to a higher prevalence of CL(P) and to higher frequency of the most severe clinical phenotypes (non-isolated cases: MMC and syndromes) and this was evaluated – and verified – in the IPDTC

registries study. The total proportion of the most severe clinical phenotypes (MMC plus cases with syndromes) by CL(P) dysmorphological severity (CL unilateral, CL bilateral, CLP unilateral and CLP bilateral) was 14.4, 23.4, 25.8 and 35.3% respectively. The odds ratio of the association between severe clinical phenotype (non-isolated cases) and dysmorphological severity versus CL unilateral was 1.81 (95% CI 1.11–2.93) for CL bilateral, 2.06 (95% CI 1.66–2.56) for CLP unilateral,

Sample size	Population characteristics	Male %	Prevalence	CLP %	CL %	CP %
6 newborn/6 years	mainly Saudis	–	0.3/1,000 live births	–	–	–
1,055/10 years 60/year from SA 19/year from Riyadh	95% Saudis	56	0.2/1,000 0.23/1,000 live births	47.8	15.7	36.5
137	all Saudis	–	2.19/1,000 live births	45	41	14
13	73% Arabs	–	0.5/1,000 births	–	–	–
563	all Omanis	50	1.5/1,000 live births	41	23	36
14	93% Omanis	–	1.5/1,000 births	35	21	43
2,146	all Jordanians	54	1.39/1,000 live births	48	30	22
60	all Jordanians	75	2.4/1,000 live births	45	20	15

and 3.24 (95% CI 2.53–4.15) for CLP bilateral (OR trend $p < 0.001$). This finding was observed even at area level, especially where the sample size was large enough.

Correlation of Prevalence Rates with Latitude and Longitude

Epidemiological studies reveal consistently wide geographic and ethnic variability of CL(P) prevalence rates. In order to evaluate this variability,

the correlation between 34 registries in Europe with latitude was examined in the IPDTC study [10]. A statistically significant correlation was evident for the total cases ($p < 0.01$) and for the non-syndromic cases ($p < 0.01$) but not for multi-malformed cases and syndromes (individually or together). No correlation was found with longitude in Europe. In 14 North American and Canadian registries there was no correlation with latitude but a slight although not statistically significant

correlation with longitude for all cases ($p = 0.14$) as well as for isolated cases ($p = 0.09$) [62].

Food Fortification with Folic Acid and Multivitamins

The role of dietary or supplemental intake of folic acid in human orofacial clefts is uncertain. Case-control studies of folic-acid containing multivitamin supplements [63–68] maternal dietary folate intake [64, 67, 69–71] and red cell and plasma folate [72–75] are inconsistent. There is some evidence that periconceptional multivitamin supplementation can reduce the birth prevalence of orofacial clefts by 30–50% [64, 76, 77]. In North America, where there has been mandatory fortification of grains with folic acid since the late 1990s, there is some evidence of a decline in the prevalence at birth of cleft lip with or without cleft palate [78, 79]. In the USA a 12% decline in cleft palate alone and a 5% decline in cleft lip with or without cleft palate following folic acid food fortification was reported by the National Birth Defects Prevention Network [79] and a report from CDC Atlanta, of a 6% fall in prevalence of total orofacial clefts [61]. However, a similar reduction has not been observed in Canada [80], Chile [81] or Australia, where there was voluntary fortification [82] – though it should be noted that the impact of voluntary fortification is unknown. Folic acid food fortification has spread rapidly in the past 5 years, especially in the Middle East, but the prevalence of timely multivitamin supplementation in the periconceptional period and early pregnancy is unknown.

Mortality due to Orofacial Clefts

With Modern Care

Surgical repair for OFC has been available in high-income settings for over 100 years. Czeizel and Sankaranarayanan [83] reported OFC outcomes in Hungary in the 1970s as 3% infant mortality and 20% survive with disability – as did Tennant et al. [84] in 2010 reporting 1% and 15% respectively. However, long-term survival is less than the

norm. Christensen et al. [85] observed increased all-cause mortality at all ages in survivors with orofacial clefts in Sweden (standardised mortality ratio = 1.4 at all ages) (see fig. 2).

Inadequate Access to Care

Cleft lip and palate exemplifies health inequality in the modern world. The available evidence suggests that in the absence of any intervention it is likely that neonatal mortality is very high, except with the mildest defect. A recent Indian study [B. Daver, pers. commun., 2007] found that in tribal areas of Maharashtra, ‘children with cleft deformities all died within a few days of birth – they had been put to the breast but since they could not suckle they died of starvation. Spoon-feeding was unheard of and there were no visiting doctors or health workers to tell parents how to feed the infants’. It is suspected that in many parts of rural India, the birth of an infant with a highly disfiguring congenital malformation leads to purposeful neglect. This may also be the case in other parts of the developing world, for example in parts of Sub-Saharan Africa where there is significant poverty and deprivation. A great deal of variation also exists in levels of education, literacy and dissemination of public health policy. In India, it has been estimated that 75% of persons with disabilities live in rural areas, 49% of them are literate yet only 34% are employed [86].

With Supportive Care Only

Where only supportive care is feasible, its extent and effectiveness is likely to be strongly influenced by culture. Even when primary surgery is available or when care is attempted, some infants still die from aspiration pneumonia or malnutrition. The best available data on survival in this situation comes from the Smile Train data for India summarised in table 4. Analysis of Smile Train data indicate that by 2010 Smile Train provided operations for 29% of affected children born in this period – a figure that corresponds with the proportion of the population urbanised. The implication is that 29% of the OFC population (of

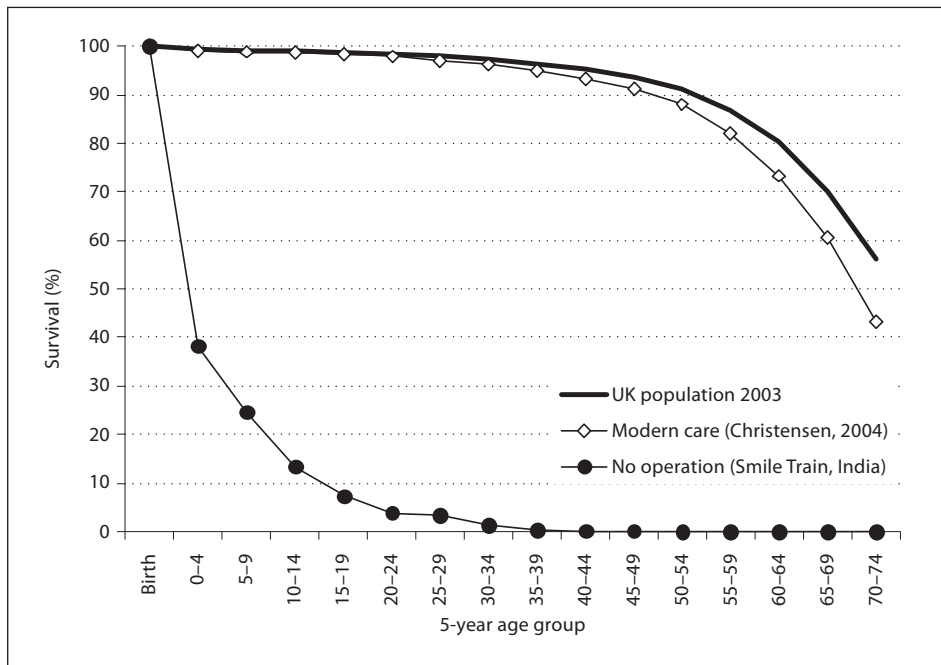


Fig. 2. The upper curve shows long-term survival of patients with orofacial clefts repaired in infancy: there is modestly increased all-cause mortality at all ages. The lower curve shows estimated survival with unoperated orofacial clefts in India, based on Smile Train data.

Table 4. Smile Train data from India: operations for OFCs by year of birth

Age 2000	Year of birth	Expected births with OFC	% of expected operated	% of 29% = unoperated survival
	2005-9	182,763	28.8	100.1
	2000-4	182,763	21.6	75.1
0-4	1995-9	182,763	11.0	38.2
5-9	1990-4	195,652	7.1	24.6
10-14	1985-9	207,038	3.9	13.4
15-19	1980-4	183,722	2.1	7.3
20-24	1975-9	151,980	1.1	3.9
25-29	1970-4	137,243	0.9	3.3
30-34	1965-9	126,630	0.4	1.5
35-39	1960-4	110,186	0.1	0.4
40-44	1955-9	94,587	0.04	0.2
45-49	1950-4	78,760	0.03	0.1
50-54	1945-9	65,530	0.02	0.1
Total		1,899,618	7.5	

any age) had access to operation. Therefore the age distribution of those who actually had an operation should reflect the age distribution of surviving patients. This proportion is shown (highlighted bold) in the final column of table 4. These data indicate that in urban areas, before the intervention of Smile Train, 38% of children with orofacial clefts survived to 5 years. However, the proportion of survivors in older age groups is far lower than this. The difference may reflect higher under-5 mortality in the recent past, or persisting increased mortality at older ages. The Smile Train data thus provides strong evidence that primary surgery is life-saving in the majority of cases. Figure 2 compares the survival curves for patients operated in childhood in a high-income situation based on Christensen et al. [85] with the survival curve for unoperated patients in a low-income setting based on Smile Train data.

Quantifying the Morbidity due to OFC

A national study of care and outcomes in children born with a unilateral cleft lip and palate (UCLP) was performed in the 1990s in the UK. The findings included poor dental arch relations in nearly 40% of 5- and 12-year-olds and 70% of 12-year-olds had midface retrusion. 15% of 12-year-olds had not received an alveolar bone graft, and 42% of bone grafts were seriously deficient or failed. 20% of 12-year-olds and 40% of 5-year-olds had untreated dental caries. Less than one-third of subjects had a good facial appearance as judged by a panel of experts [87]. Assessment of speech outcome found that 19% of 5-year-olds and 4% of 12-year-olds were judged to be impossible to understand or just intelligible to strangers. 34% of 5-year-olds and 17% of 12-year-olds had at least one serious error of consonant production. 18% of 5- and 12-year-olds had consistent hypernasality of mild, moderate, or severe degree. Approximately two-thirds of both age groups had undergone speech therapy [88].

In India where unoperated adult cleft lip and palate is a significant problem, very few achieved

normal speech [86], and communication is hampered by hearing problems with 76% of unoperated cleft palate patients showing mild to moderate conductive deafness [89].

The psychosocial impact in OFC is not easy to define and quantify, and is not closely correlated with the severity of the problem, but the finding of increased adult suicide rates in Denmark [85] and the high school drop-out rate, unemployment, behavioural problems, episodes of depression and low self-esteem [86] are indications of the need for this to be very seriously considered in addressing the problem of OFC.

The WHO GBD project seeks to examine both mortality and morbidity, and has found evidence of tremendous inequalities in relation to the setting, such that with modern surgery and support the survival rate at age 15–19 were 98.5%, but with no care this is only 7.3%. In terms of morbidity, three sequela groups are considered with their estimated distribution according to whether patients are operated or not, and for those that are, whether there is a residual disability. For unoperated orofacial clefts, those that survive have a prevalence of residual disability as defined by WHO is 100%, but even for those with modern surgery this is 15–25% depending on whether multidisciplinary care is available. The majority of orofacial clefts are of course effectively cured (quality of life = population norm), and this illustrates the value of surgery as a life-saving and rehabilitative procedure. Significant questions however remain about quality of life and psychosocial effects which are difficult to quantify. In fact, the social impact of congenital disorders increases with age much more than their clinical effect. The final burden of the disorder can only be assessed by the social situation of surviving mature adults.

Conclusions

The taxonomy of disease is becoming redefined as the understanding of disease mechanisms at a molecular level improves and as we acquire a

greater understanding of the interactions between an individual's genetic background and their environment. Descriptive epidemiology in the field of orofacial clefts is an excellent example of how observation of the patterns and trends of the presentation of a disease, paying particular attention to phenotype and environment, is underpinning research hypothesis and fuelling further discovery. It is widely acknowledged that non-syndromic, non-chromosomal orofacial clefting is a disease with a polygenic multifactorial aetiology that exhibits geographic, ethnic and racial variations in its prevalence but also there are very interesting variations emerging on the basis of sub-phenotype, gender and exposure to environmental variables as described above. Further investigation of such

factors at a population level, and ultimately at an individual level with the support of birth defects surveillance systems and evolving knowledge of genetic mutations in disease causality [90] will eventually lead to the development of strategies for primary prevention.

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References

- Mossey PA, Little J: Epidemiology of oral clefts: an international perspective; in Wyszynski DF (ed): *Cleft Lip and Palate. From Origin to Treatment*. New York, Oxford University Press, 2002, chapt 12, pp 127–158.
- Global Registry and Database on Craniofacial Anomalies. WHO Human Genetics Programme: Management of Noncommunicable Diseases: International Collaborative Research on Craniofacial Anomalies. Geneva, WHO, 2003.
- Croen LA, Shaw GM, Wasserman CR, Tolarova MM: Racial and ethnic variations in the prevalence of orofacial clefts in California, 1983–1992. *Am J Med Genet* 1998;79:42–47.
- Kirby R, Petrini J, Alter C: Collecting and interpreting birth defects surveillance data by Hispanic ethnicity: a comparative study. *The Hispanic Ethnicity Birth Defects Workgroup. Teratology* 2000;61:21–27.
- Leck I, Lancashire RJ: Birth prevalence of malformations in members of different ethnic groups and in the offspring of matings between them, in Birmingham, England. *J Epidemiol Community Health* 1995;49:171–179.
- Ching GHS, Chung CS: A genetic study of cleft lip and palate in Hawaii. I. Interracial crosses. *Am J Hum Genet* 1974;26:162–172.
- Tolarova MM, Cervenka J: Classification and birth prevalence of orofacial clefts. *Am J Med Genet* 1998;75:126–137.
- Hagberg C, Larson O, Milerad J: Incidence of cleft lip and palate and risks of additional malformations. *Cleft Palate Craniofac J* 1997;35:40–45.
- Online Mendelian Inheritance in Man, OMIM®. Baltimore, McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University, 2000. www URL: <http://omim.org/>
- IPDTC Working Group: Prevalence at birth of cleft lip with or without cleft palate: data from the International Perinatal Database of Typical Oral Clefts (IPDTC). 2011;48:66–78.
- Rittler M, Lopez-Camelo JS, Castilla EE, Bermejo E, Cocchi G, Correa A, Csaky-Szunyogh M, Danderfer R, De Vigan C, De Walle H, Da Grace Dutra M, Hirahara F, Martinez-Frias ML, Merlob P, Mutchinick O, Ritvanen A, et al: Preferential associations between oral clefts and other major congenital anomalies. *Cleft Palate Craniofac J* 2008;45:525–532.
- Rittler M, Cosentino V, Lopez-Camelo JS, Murray JC, Wehby G, Castilla EE: Associated anomalies among infants with oral clefts at birth and during a 1-year follow-up. *Am J Med Genet Part A* 2011;155A:1588–1596.
- Grosen D, Bille C, Pedersen JK, Skytthe A, Murray JC, Christensen K: Recurrence risk for offspring of twins discordant for oral cleft – a population-based cohort study of the Danish 1936–2004 cleft twin cohort. *Am J Med Genet A* 2011;152A:2468–2474.
- Fogh-Anderson P: PhD Thesis, Copenhagen 1942.
- Fraser GR, Calnan JS: Cleft lip and palate: seasonal incidence, birth weight, birth rank, sex, site, associated malformations and parental age. *Arch Dis Child* 1961;36:420–423.
- Bonaiti C, Briard ML, Feingold J, Pavy B, Psaume J, Migne-Tufferaud G, Kaplan J: An epidemiological and genetic study of facial clefting in France: epidemiology and frequency in relatives. *J Med Genet* 1982;19:8–15.
- Tolarova MM: Orofacial clefts in Czechoslovakia: incidence, genetics and prevention of cleft lip and palate over a 19-year period. *Scand J Plast Reconstr Surg* 1987;21:19–25.
- Jensen BL, Kreiborg S, Dahl E, Fogh-Andersen P: Cleft lip and palate in Denmark 1976–1981: epidemiology variability and early somatic development. *Cleft Palate J* 1988;25:258–269.
- Johnston MC, Brown KS: Human population data. General discussion. III. *Prog Clin Biol Res* 1980;1.

- 20 Mossey PA, Little J, Munger RG, Dixon MJ, Shaw WC: Cleft lip and palate. *Lancet* 2009;374:1773–1785.
- 21 Fujino H, Tanaka K, Sanui Y: Genetic study of cleft lips and cleft palates based upon 2,828 Japanese cases. *Kyushu J Med Sci* 1963;14:317–331.
- 22 Niswander JD, MacLean CJ, Chung CS, Dronamraju K: Sex ratio and cleft lip with or without cleft palate. *Lancet* 1972;ii:858–860.
- 23 Burdi AR, Silvey RG: Sexual differences in closure of the human palatal shelves. *Cleft Palate J* 1969;6:1–7.
- 24 Harville EW, Wilcox AJ, Lie RT, Vindenes H, Abyholm F: Cleft lip and palate versus cleft lip only: are they distinct defects? *Am J Epidemiol* 2005;162:448–453.
- 25 Calzolari E, Pierini A, Astolfi G, Bianchi F, Neville AJ, Rivieri F: Associated anomalies in multimalformed infants with cleft lip and palate: an epidemiologic study of nearly six million births in twenty-three EUROCAT registries. *Am J Med Genet A* 2007;143A:528–537.
- 26 Dolk H: EUROCAT: 25 years of European surveillance of congenital anomalies. *Arch Dis Child Fetal Neonatal Ed* 2005;90:F355–F358.
- 27 Vandas AP: Incidence of cleft lip, cleft palate, and cleft lip and palate among races: a review. *Cleft Palate J* 1987; 24:216–225.
- 28 Milerad J, Larson O, Hagberg C, Ideberg M: Associated malformations in infants with cleft lip and palate: a prospective, population-based study. *Pediatrics* 1997;100:180–186.
- 29 Shafi T, Khan MR, Atiq M: Congenital heart disease and associated malformations in children with cleft lip and palate in Pakistan. *Br J Plast Surg* 2003;56:106–109.
- 30 Shaw GM, Carmichael SL, Yang W, Harris JA, Lammer EJ: Congenital malformations in births with orofacial clefts among 3.6 million California births, 1983–1997. *Am J Med Genet Part A* 2004;125A:250–256.
- 31 Stoll C, Alembik Y, Dott B, Roth MP: Associated malformations in cases with oral clefts. *Cleft Palate Craniofac J* 2000;37:41–47.
- 32 Rawashdeh MA, Jawdat Abu-Hawas B: Congenital associated malformations in a sample of Jordanian patients with cleft lip and palate. *J Oral Maxillofac Surg* 2008;66:2035–2041.
- 33 Kallen B, Harris J, Robert E: The epidemiology of orofacial clefts. 2. Associated malformations. *J Craniofac Genet Dev Biol* 1996;16:242–248.
- 34 Genisca AE, Frias JL, Broussard CS, Honein MA, Lammer EJ, Moore CA, Shaw GM, Murray JC, Yang W, Rasmussen SA: Orofacial clefts in The National Birth Defects Prevention Study (1997–2004). *Am J Med Genet A* 2009;149A:1149–1158.
- 35 Iregbulem LM: The incidence of cleft lip and palate in Nigeria. *Cleft Palate J* 1982;19:201–205.
- 36 Ogle OE: Incidence of cleft lip and palate in a newborn Zairian sample. *Cleft Palate Craniofac J* 1993;30:250–251.
- 37 Khan AA: Congenital malformation in African neonates in Nairobi. *J Trop Med Hyg* 1965;68:272.
- 38 Simpkins M, Lowe A: Congenital abnormalities in the African newborn. *Arch Dis Child* 1961;36:404–405.
- 39 Gupta B: Incidence of congenital malformation in Nigerian children. *West Afr Med J* 1969;18:22–27.
- 40 Robinson DC, Shepherd JJ: The prevalence and natural history of cleft lip and palate in Uganda. *Dev Med Child Neurol* 1970;12:636–641.
- 41 Khrouf N, Spang R, Podgorna T, Miled SB, Moussaoui M, Chibani M: Malformations in 10,000 consecutive births in Tunis. *Acta Paediatr Scand* 1986;75:534–539.
- 42 Kromberg JGR, Jenkins T: Common birth defects in South African Blacks. *S Afr Med J* 1982;62.
- 43 Morrison G, Cronje AS, Van Vuuren I, Op't Hof J: The incidence of cleft lip and palate in the Western Cape. *S Afr Med J* 1985;68:576–577.
- 44 Butali A, Mossey PA: Epidemiology of orofacial clefts in Africa: methodological challenges in ascertainment. *Pan Afr Med J* 2009;2:1–11.
- 45 Myrianthopoulos NC, Chung CS: Congenital malformations in singletons: epidemiological survey. Report from the Collaborative Perinatal Project. New York, Stratton Intercontinental Medical Book Corp, 1974.
- 46 Wyszynski DE, Beaty TH, Maestri N: Genetics of non-syndromic cleft lip with or without cleft palate revisited. *Cleft Palate Craniofac J* 1996;33:406–417.
- 47 Das SK, Runnels RS, Smith JC, Cohly HH: Epidemiology of cleft lip and cleft palate in Mississippi. *South Med J* 1995;88:437–442.
- 48 Al-Johar A, Ravichandran K, Subhani S: Pattern of cleft lip and palate in hospital-based population in Saudi Arabia: retrospective study. *Cleft Palate Craniofac J* 2008;45:592–596.
- 49 Kumar P, Hussain MT, Cardoso E, Hawary MB, Hassanain J: Facial clefts in Saudi Arabia: an epidemiologic analysis in 179 patients. *Plast Reconstr Surg* 1991;88:955–958.
- 50 Borkar AS: Epidemiology of facial clefts in the central province of Saudi Arabia. *Br J Plast Surg* 1993;46:673–675.
- 51 Aqrabawi HE: Facial cleft and associated anomalies: incidence among infants at a Jordanian medical centre. *East Mediterr Health J* 2008;14:356–359.
- 52 Rajab A, Thomas C: Oral clefts in the Sultanate of Oman. *Eur J Plast Surg* 2001;24:230–233.
- 53 Sabbagh HJ, Mossey PA, Innes NP: Prevalence of orofacial clefts in Saudi Arabia and neighboring countries: a systematic review. *Saudi Dent J* 2012;24:3–10.
- 54 Smahel Z, Brejcha M: Differences in craniofacial morphology between complete and incomplete unilateral cleft lip and palate in adults. *Cleft Palate J* 1983;20:113–127.
- 55 Nordin KE, Larson O, Nylén B, Eklund G: Early bone grafting in complete cleft lip and palate cases following maxillofacial orthopedics. I. The method and skeletal development from seven to thirteen years of age. *Scand J Plast Reconstr Surg* 1983;17:33–50.
- 56 Semb G, Shaw WC: Simonart's band and facial growth in unilateral clefts of the lip and palate. *Cleft Palate Craniofac J* 1991;28:40–48.
- 57 Roberts-Harry D, Semb G, Hathorn I, Killingback N: Facial growth in patients with unilateral clefts of the lip and palate: a two-center study. *Cleft Palate Craniofac J* 1996;33:489–493.

- 58 Da Silva Filho O, Santamaria M, Da Silva Dalben G, Semb G: Prevalence of a Simonart's band in patients with complete cleft lip and alveolus and complete cleft lip and palate. *Cleft Palate Craniofac J* 2006;43:442–445.
- 59 Rahimov F, Marazita ML, Visel A, Cooper ME, Hitchler MJ, Rubini M, Domann FE, Govil M, Christensen K, Bille C, Melbye M, Jugessur A, Lie RT, Wilcox AJ, Fitzpatrick DR, Green ED, Mossey PA, Little J, Steegers-Theunissen RP, Pennacchio LA, Schutte BC, Murray JC: Disruption of an AP-2a binding site in an IRF6 enhancer is associated with cleft lip. *Nat Genet* 2008;40:1341–1347.
- 60 Forrester MB, Merz RD, Yoon PW: Impact of prenatal diagnosis and elective termination on the prevalence of selected birth defects in Hawaii. *Am J Epidemiol* 1998;148:1206–1211.
- 61 Yazdy M, Honein M, Xing J: Reduction in orofacial clefts following folic acid fortification of the US grain supply. *Birth Defects Res A Clin Mol Teratol* 2007;79:16–23.
- 62 Mossey PA: Epidemiology underpinning research in the etiology of orofacial clefts. *Orthod Craniofac Res* 2007;10:114–120.
- 63 Hill L, Murphy M, McDowall M, Paul AH, et al: Maternal drug histories and congenital malformations: limb reduction defects and oral clefts. *J Epidemiol Community Health* 1988;42:1–7.
- 64 Shaw GM, Lammer EJ, Wasserman CR, et al: Risks of orofacial clefts in children born to women using multivitamins containing folic acid periconceptionally. *Lancet* 1995;346:393–396.
- 65 Czeizel AE, Timar L, Sarkozi A, et al: Dose-dependent effect of folic acid on the prevention of orofacial clefts. *Pediatrics* 1999;104:e66.
- 66 Kallen B: Maternal drug use and infant cleft lip/palate with special reference to corticoids. *Cleft Palate Craniofac J* 2003;40:624–628.
- 67 Van Rooij IA, Ocke MC, Straatman H, et al: Periconceptional folate intake by supplement and food reduces the risk of nonsyndromic cleft lip with or without cleft palate. *Prev Med* 2004;39:689–694.
- 68 Badovinac RL, Werler MM, Williams PL, Kelsey KT, Hayes C: Folic acid-containing supplement consumption during pregnancy and risk for oral clefts: a meta-analysis. *Birth Defects Res A Clin Mol Teratol* 2007;79:8–15.
- 69 Hayes C, Werler MM, Willett WC, et al: Case-control study of periconceptional folic acid supplementation and orofacial clefts. *Am J Epidemiol* 1996;143:1229–1234.
- 70 Krapels IP, van Rooij IA, Ocke MC, West CE, van der Horst CM, Steegers-Theunissen RP: Maternal nutritional status and the risk for orofacial cleft offspring in humans. *J Nutr* 2004;134:3106–3113.
- 71 Little J, Gilmour M, Mossey PA, Fitzpatrick D, Cardy A, Clayton-Smith J, Fryer AE: ITS MAGIC Collaboration. Folate and clefts of the lip and palate – a UK-based case-control study. Part I: Dietary and supplemental folate. *Cleft Palate Craniofac J* 2008;45:420–427.
- 72 Wong WY, Eskes TKAB, Kuijpers-Jagtman AM, Spauwen PHM, Steegers EAP, Thomas CMG, et al: Nonsyndromic orofacial clefts: association with maternal hyperhomocysteinemia. *Teratology* 1999;60:253–257.
- 73 Van Rooij IA, Vermeij-Keers C, Kluijtmans LA, et al: Does the interaction between maternal folate intake and the methylenetetrahydrofolate reductase polymorphisms affect the risk of cleft lip with or without cleft palate? *Am J Epidemiol* 2003;157:583–591.
- 74 Munger RG, Sauberlich HE, Corcoran C, Nepomuceno B, Daack-Hirsch S, Solon FS: Maternal vitamin B6 and folate status and risk of oral cleft birth defects in the Philippines. *Birth Defects Res A Clin Mol Teratol* 2004;70:464–471.
- 75 Little J, Gilmour M, Mossey PA, Fitzpatrick D, Cardy A, Clayton-Smith J, Hill A, Duthie SJ, Fryer AE, Molloy AM, Scott JM: ITS MAGIC Collaboration. Folate and clefts of the lip and palate – a UK-based case-control study. Part II: Biochemical and genetic analysis. *Cleft Palate Craniofac J* 2008;45:428–438.
- 76 Loffredo L, Souza JMP, Freitas JAS, Mossey PA: Oral clefts and vitamin supplementation. *Cleft Palate Craniofac J* 2000;37:69–76.
- 77 Itikala PR, Watkins ML, Mulinare J, Moore CA, Liu Y: Maternal multivitamin use and orofacial clefts in the offspring. *Teratology* 2001;63:79–86.
- 78 Simmons CJ, Mosley BS, Fulton-Bond CA, Hobbs CA: Birth defects in Arkansas: is folic acid fortification making a difference? *Birth Defects Res A Clin Mol Teratol* 2004;70:559–564.
- 79 Canfield MA, Collins JS, Botto LD, Williams LJ, Mai CT, Kirby RS, et al: Changes in the birth prevalence of selected birth defects after grain fortification with folic acid in the United States: findings from a multi-state population-based study. *Birth Defects Res A Clin Mol Teratol* 2005;73:679–689.
- 80 Ray JG, Meier C, Vermeulen MJ, Wyatt PR, Cole DE: Association between folic acid food fortification and congenital orofacial clefts. *J Pediatr* 2003;143:805–807.
- 81 López-Camelo JS, Orioli IM, da Graça Dutra M, Nazer-Herrera J, Rivera N, Ojeda ME, Canessa A, Wettig E, Fontanaz AM, Mellado C, Castilla EE: Reduction of birth prevalence rates of neural tube defects after folic acid fortification in Chile. *Am J Med Genet A* 2005;135:120–125.
- 82 Botto LD, Lisi A, Bower C, Canfield MA, Dattani N, De Vigan C, et al: Trends of selected malformations in relation to folic acid recommendations and fortification: an international assessment. *Birth Defects Res A Clin Mol Teratol* 2006;76:693–705.
- 83 Czeizel A, Sankaranarayanan K: The load of genetic and partly genetic disorders in man. Congenital anomalies: estimates of detriment in terms of life years lost and years of impairment. *Mutat Res* 1984;128:73–103.
- 84 Tennant PW, Pearce MS, Bythell M, Rankin J: Twenty-year survival of children born with congenital anomalies: a population-based study. *Lancet* 2010;375:649–656.
- 85 Christensen K, Juel K, Herskind AM, Murray JC: Long-term follow up study of survival associated with cleft lip and palate at birth. *BMJ* 2004;328:1405–1411.
- 86 Murthy J: Management of cleft lip and palate in adults. *Indian J Plast Surg* 2009;42:116–122.

- 87 Williams AC, Bearn D, Mildinhall S, Murphy T, Sell D, Shaw WC, Murray JJ, Sandy JR: Cleft lip and palate care in the United Kingdom – the Clinical Standards Advisory Group (CSAG) Study. Part 2: Dentofacial outcomes and patient satisfaction. *Cleft Palate Craniofac J* 2001;38:24–29.
- 88 Sell D, Grunwell P, Mildinhall S, Murphy T, Cornish TA, Bearn D, Shaw WC, Murray JJ, Williams AC, Sandy JR: Cleft lip and palate care in the United Kingdom – the Clinical Standards Advisory Group (CSAG) Study. Part 3: Speech outcomes. *Cleft Palate Craniofac J* 2001;38:30–37.
- 89 Ramana YV, Nanda V, Biswas G, Chittoria R, Ghosh S, Sharma RK: Audiological profile in older children and adolescents with unrepaired cleft palate. *Cleft Palate Craniofac J* 2005;42:570–573.
- 90 Cotton RG: Rare disease registries and mutation/variation databases. *Hum Mutat* 2011;32:1073–1074.

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Genetic and Environmental Factors in Human Cleft Lip and Palate

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Abstract

Cleft lip and palate is the most common craniofacial birth defect and its etiology has been the focus of many reports in the literature. It is well accepted that both genetics and environment play a role in the condition, however we still have not been able to translate what have been learned into clinical applications. This paper provides an interpretation of the latest research findings in humans and a perspective for where the field is going. The latest effort in gene identification and the associations between isolated cleft lip and palate and the loci harboring *IRF6* (1q32) and 8q24.21 are highlighted, as well as the latest insight from more sophisticated phenotypical characterization and the inclusion of covariables related to the environment in the analysis of genetic variation.

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It was just a few weeks before I was born that Dr. F. Clark Fraser published a review paper highlighting the conclusions of a workshop on cleft lip and palate sponsored by the National Institutes of Health of the United States [1]. The goal was to review evidence for and against multifactorial inheritance and other concepts of causation, to decide what further data were needed, and to consider possible applications of these concepts to the practical problems of family counseling and prevention of these defects. Dr. Fraser predicted: *If*

the etiology is indeed multifactorial. . .the effects of the underlying genes will be harder to distinguish than they are in conditions showing simple modes of inheritance. There will probably be no identifiable biochemical defect. After 40 years, much has been learned, but we have not identified a mechanism (a biochemical defect) that has allowed the translation of bench results into changes in clinical practice. In this review, the latest achievements in the understanding of cleft lip and palate etiology in humans are reviewed as well as current efforts toward further discovery.

Gene Identification

Since the first publication of association between a candidate gene (transforming growth factor- α or *TGFA*) and isolated cleft lip and palate in 1989 [2], many publications have described similar attempts to identify further cleft genes [3]. As for the original work developed under the leadership of Dr. Jeffrey C. Murray [2], work done during the 1990s was guided by segregation analyses that suggested that one-third of isolated cleft lip and palate cases are explained by a single major gene model [4, 5]. In the original work, Dr. Murray and colleagues highlighted that although association

studies are the first step to facilitate identification of susceptibility alleles for isolated cleft lip and palate, more work was needed to allow for (1) more accurate estimates of haplotype frequencies (due to the potential inflation of the frequency derived from homozygosity and small sample sizes), (2) replication of the results in other populations, and (3) since this type of data does not allow for the determination of the overall contribution of a particular locus, generating evidence from linkage would substantiate the association, as well as indicate that the locus would indeed fit the major gene model [2]. What we witnessed afterwards were several reports that did not necessarily corroborate the association between *TGFA* and cleft lip and palate [6]. In the last decade we have moved from smaller scale candidate gene work to larger scale genome-wide approaches, but one interesting detail is that association studies are not confirmed by linkage approaches and vice versa [3]. In the work providing the first evidence that *IRF6* (interferon regulatory factor 6) is associated with clefts [7], more than 8,000 DNA samples were used and association between a single nucleotide polymorphism (SNP) and isolated cleft lip and palate was conclusively shown, with the results particularly driven by a Filipino cohort. A number of papers followed replicating this association in other populations [3]. However, when genome-wide linkage and more dense candidate gene analyses were performed in an overlapping group of samples from the Philippines, statistically significant linkage and association between *IRF6* and isolated cleft lip and palate were not present, even though borderline results in markers 10–20 million base pairs upstream and downstream of *IRF6* could be seen if one was willing to overlook the traditional threshold of $\alpha = 0.05$ (fig. 1) [8, 9]. Additionally, association between isolated cleft lip and palate and *IRF6* in populations that initially showed negative results (i.e. South Americans) can be seen when analysis is done discriminating by population-specific genetic markers [10]. The simplest explanation for this phenomenon is that

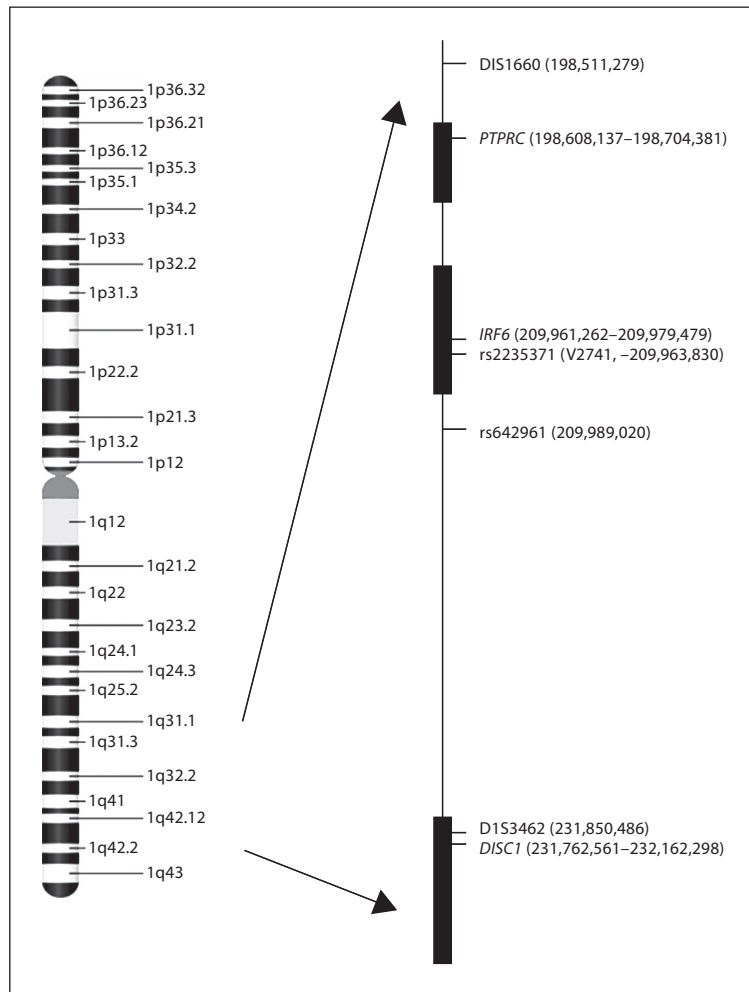
the major gene model does not really explain one-third of isolated cleft lip and palate inheritance but likely much less, and a polygenic model with several genes with small effects may be the most common inheritance mode related to isolated cleft lip and palate.

Genome-wide searches have been utilized with genetic marker panels varying from a few hundred to one million variants. A number of family-based linkage studies have been published [8, 11–22] but the results do not clearly overlap, emphasizing the assumption that a major gene effect for isolated cleft lip and palate does not explain a substantial number of cases (table 1). More robust association studies also started to appear in the literature, identifying a novel locus for isolated cleft lip and palate in 8q24 (table 2) [23–25], additional ones in 10q25.3, 17q22 [26], 1q22.1, and 20q12 [25] and confirming the association with *IRF6* [25]. Unveiling the association between isolated cleft lip and palate and 8q24 is remarkable because the associated SNPs lie on a gene desert region (fig. 2). In other words, candidate gene/loci approaches would never have detected this signal. The other remarkable fact is that a number of nearby SNPs have been associated with several types of cancer and other complex conditions [27–42]. This physical proximity is at least intriguing since there is a suggestion that individuals born with clefts may have a higher risk for certain types of cancer [43, 44] and their unaffected relatives may also have increased risks for cancer [45].

Redefining Isolated Cleft Lip and Palate

With the evidence that no major gene effect is increasing the susceptibility to isolated cleft lip and palate in most cases, sample sizes needed to ensure that there is adequate power to identify association at the required significance threshold are probably very large for most of the contributing genes. As an example, if we consider that a susceptibility allele has a frequency 1% higher in cleft

Fig. 1. Chromosome 1 locus. This locus showed both association and linkage to cleft lip and palate but the results are hard to interpret. V274I (rs2235371) is the marker in the original *IRF6* study [7] that showed the strongest association with cleft lip and palate. D1S1660 (1q31.1) and D1S3462 (1q42.2) had suggestive linkage results (LOD scores 2.17 and 2.85, respectively) to cleft lip and palate in a subset of the same Filipino cohort used in the original study [8]. The marker D1S1660 is upstream to *PTPRC* (protein tyrosine phosphatase, receptor type, C) and D1S3462 is in *DISC1* (disrupted in schizophrenia 1 isoform). Since V274I was not informative in Europeans, further work in the region suggested that rs642961 was not only associated with both Filipinos and Danish, but could also be the susceptibility allele for isolated cleft lip and palate [73]. Later reports suggested that rs642961 is not the allele increasing susceptibility to isolated cleft lip and palate, particularly in population groups not from the Philippines or Denmark [74, 75]. It is still not clear what is (are) the susceptibility allele(s) in this locus and currently no clinical application can be suggested from these findings. Base pair locations are based on the UCSC Genome Browser on Human February 2009 assembly.



cases in comparison to controls, and assuming we can have 3 times the number of controls in comparison to cases, we would need 5,600 cases and 16,700 controls to achieve 80% statistical power. Not only do these estimates suggest individual investigators are unlikely to gather these sample sizes in a relatively short period of time (the identification of *IRF6* was the result of a joint effort of a group of investigators organized by Dr. Jeffrey C. Murray who were recruiting subjects individually for more than a decade [7]), but also it is

likely that many contributing genes will never be identified.

To address concerns related to heterogeneity and sample size, study designs with more sophisticated clinical descriptions to further characterize individuals born with clefts have been proposed. While these assumptions may help identify only some of the relevant risk alleles, initial findings are promising [8, 9]. Dental anomalies have been one of the traits proposed to further characterize oral clefts [46]. Individuals born

Table 1. Genome-wide linkage studies for isolated cleft lip and palate

Population studied	Number of families studied	Number of markers genotyped	Linked loci highlighted in the original publications
England [11]	91	400	1p, 2p, 6p, 8q, 11cen, 12q, 16p, Xcen-q
China [12]	36	387	chromosome 16
Argentina, Mexico, and USA [13]	10	368	chromosomes 2, 6, 17, 18
Syria [14]	2	368	17p13.1
India [15]	38	285	5q11, 7p15, 15q26, 20q13
Turkey [16]	18	453	chromosomes 1, 2, 3, 4, 6, 7, 9, 13, 15, 16, 17, 18, 9, 22
Meta-analysis (Argentina, Australia, China, Colombia, England, India, Mexico, Syria, Turkey, and USA) [17]	574	387	1p12–13, 6p23, 6q23–25, 9q21, 14q21–24, 15q15
India [18]	2	10,000	13q33.1–34
Philippines [19]	220	392	8p11–23
Philippines [8]	46	392	19p13.12–19q12
China, Colombia, India, Philippines, Turkey, and USA [20]	820	392	1q32, 2p13, 3q27–28, 9q21, 12p11, 14q21–24, 16q24
Germany [21]	91	542	1p21–p31, 4q21–q26
China [22]	2	382	1q32–q42, 2p24–p25

Table 2. Genome-wide association studies for isolated cleft lip and palate suggesting 8q24 is a susceptibility locus for the defect

Population studied	Sample sizes	Number of SNPs analyzed	Additional information
Germany [23]	224 cases, 383 controls	521,176	Replication was done using 462 cases (216 from the primary analysis and an additional 246 cases) and 954 controls
USA [24]	111 cases, 5,951 controls	495,858	–
13 populations [25]	1,908 case-parent trios (7,178 individuals)	589,945	Replication was done in 8,115 individuals from 1,965 families drawn from 12 populations

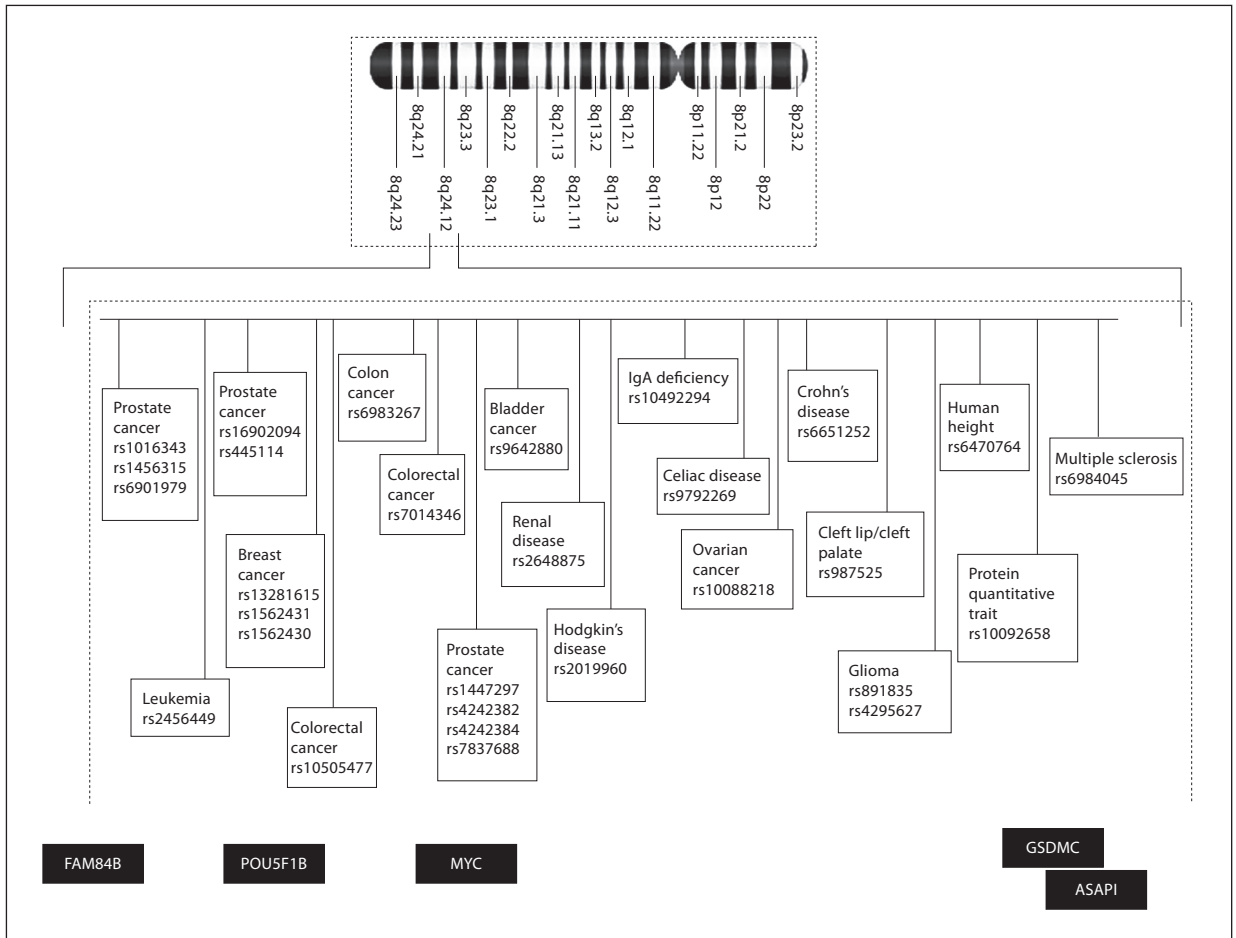


Fig. 2. Chromosome 8 locus. The 8q24.21 gene desert region lies between *MYC* (myc proto-oncogene protein) at 128,748,315 base pairs and *GSDMC* (melanoma-derived leucine zipper, extranuclear) at 130,760,443 base pairs. The association signal with isolated cleft lip and palate is at 129,946,154 base pairs (rs987525). Association signals with other complex traits are also described. Base pair locations are based on the UCSC Genome Browser on Human February 2009 assembly. *FAM84B* = Family with similarity 84, member B; *POU5F1B* = POU class 5 homeobox 1B; *ASAP1* = ArfGAP with SH3 domain, ankyrin repeat and PH domain 1.

with clefts have 3–4 times the odds of presenting dental anomalies, particularly tooth agenesis [46], and increased frequencies can also be seen in their parents and siblings [47]. One emerging pattern appears to be the presence of anomalies in the upper lateral incisor opposite the side of a cleft lip (table 3; fig. 3). This clinical presentation was suggested as being a form of bilateral cleft of the lip,

which in one of the sides is expressed as a shape anomaly or agenesis of the upper lateral incisor [46–48]. These approaches bring another interesting question: is there truly an isolated form of clefts? Variation in the morphology of the face, brain, teeth, dermatoglyphics, handedness and other features have been proposed as tools to further characterize isolated forms of clefts [49]. It is

Fig. 3. Upper arch diagram. Schematic representation of unilateral cleft (a) and unilateral cleft with agenesis of the lateral incisor (arrow) on the opposite side of the cleft (b), also referred to as ‘unsuccessful or occult cleft’. i = Central incisor; l = lateral incisor; c = canine; pm = pre-molar; m = molar.

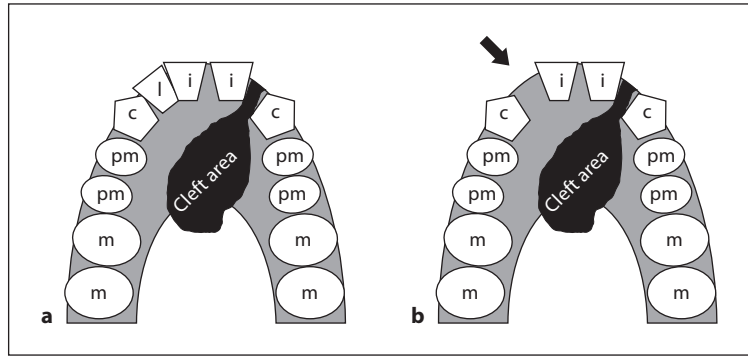


Table 3. Frequency of cleft lip and palate cases with associated dental anomalies that had anomalies of the upper lateral incisor of the opposite side of a cleft lip

Study population	Frequency	Reference
Argentina	0.125	unpubl. data
Philippines	0.26	unpubl. data
Brazil (Bauru)	0.21	46
Brazil (Rio de Janeiro)	0.39	47
USA	0.38	48

intuitive to think that isolated cleft lip and palate is in reality a syndrome or group of syndromes.

Another intriguing aspect is the presentation of unilateral cleft of the lip, which occurs in two-thirds of the instances on the left side. Are there differences in the genetic control between the left and right sides? The evidence suggests yes. Association with *AXIN2* and *CDH1* (epithelial cadherin or E-cadherin) was found for unilateral right cleft lip with dental anomalies and not for the left side [50]. Previous work showed that dental anomalies of the posterior segments of the maxilla and mandible tend to occur in the opposite side of the clefted lip [46]. It appears that gene expression patterns will differ between left and right both in the lip and in the dentition. Evidence

from many vertebrates suggest that after an initial symmetry breaking event in the jaw and with the participation of Bmps (bone morphogenetic proteins), a conserved pathway including *nodal*, *lefty* (left-right determination factor), and *Pitx2* (paired-like homeodomain transcription factor 2) operates in the left side, but a similar event has not been identified on the right [51]. Hence, we may assume that differences in side affection seen in isolated cleft lip and palate may be the result of specific gene regulation events in the left and right sides (fig. 4). The definition of clinical constellations that are associated with specific genetic factors will likely allow us to reclassify isolated cleft lip and palate in more discreet entities that will have more meaningful clinical significance. However, those will be still under the possible influence of environmental factors.

Environment

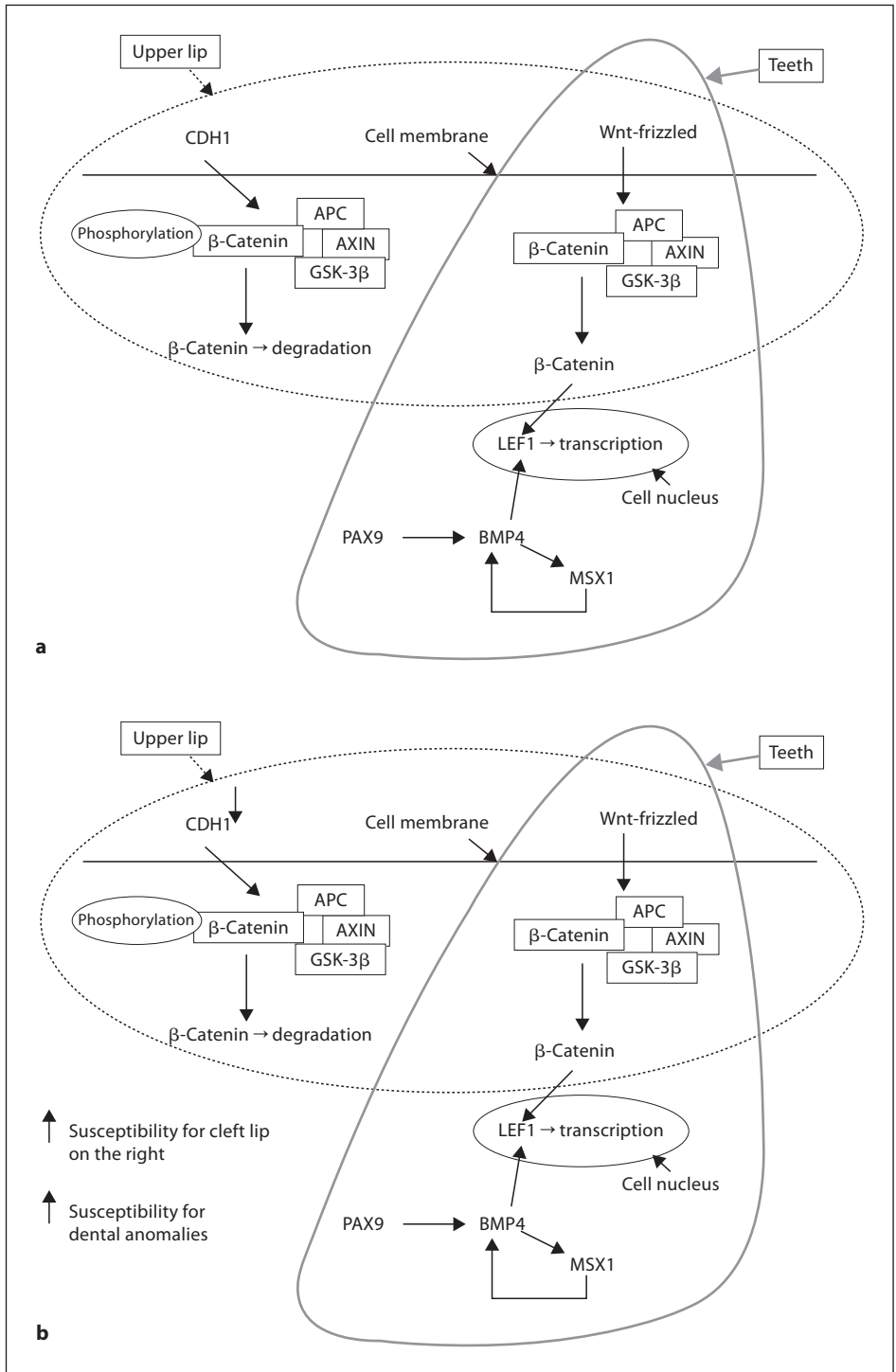
Maternal cigarette smoking, alcohol consumption and multivitamin use are the three factors most considered with regard to the risks of isolated cleft lip and palate. There is solid evidence that maternal cigarette smoking increases the risk of oral cleft in offspring [3]. Data on alcohol consumption and multivitamin use are less conclusive. The most challenging aspect of any

study investigating the influence of environmental factors on cleft susceptibility continues to be data collection. Data on these variables are usually collected after birth and in the best-case scenario several months have passed since the pre- and periconceptional periods. The most recent analysis of these kinds of data includes the evaluation of 550 cleft palate-only case-parent trios from 12 sites with genome-wide genotyping data available [52]. Borderline results suggested a possible association between markers in chromosome 9 and maternal alcohol consumption in cases of isolated cleft palate. Similarly, markers in *TBK1* (TANK-binding kinase 1) on chromosome 12 and *ZNF236* (zinc finger protein 236) on chromosome 18 had borderline association with cases of isolated cleft palate and maternal cigarette smoking. Finally, multivitamin use appeared to decrease the risk for isolated cleft palate depending on variation in *BAALC* (brain and acute leukemia, cytoplasmic) on chromosome 8.

With regard to maternal alcohol consumption, consuming five or more alcoholic drinks per sitting during the first trimester of pregnancy elevates 2.5 times the risk of oral clefts in the offspring. Furthermore, it appears that this risk is related particularly to children carrying specific genetic variation in *ADH1C* (alcohol dehydrogenase 1C) [53]. Evidence suggesting an interaction between *IRF6* and maternal multivitamin use [54] and *ZNF533* and maternal vitamin use [55] as decreasing the susceptibility to isolated cleft lip and palate has also been reported. Maternal cigarette smoking, which is the factor with most solid evidence when it comes to isolated cleft lip and palate susceptibility, is believed to increase embryonic hypoxia. One human model of hypoxia leading to oral clefting is the acardiac fetus, which represents an accident of monozygotic twinning or higher multiple births due to an artery-to-artery and a vein-to-vein anastomosis in the monochorial placenta. Blood returning to the placenta through the umbilical artery of a normal co-twin is directed into the umbilical artery of the acardiac twin,

such that blood reaching the cranial end of the embryo poorly oxygenated, resulting in a number of structural defects including oral clefts [56]. The frequency and severity of structural defects in acardiac twins are not random but occur in a gradient, increasing in severity from the caudal to the cephalic end of the fetus, reflecting the decreasing pulse pressure and deficient oxygen saturation in the reversed circulation [57]. In an analysis of 29 years of medical records, out of 12 acardiac fetuses, 6 presented oral clefts [56], providing strong evidence that hypoxia is an important contributor to cleft lip and palate and cleft palate alone in acardiac fetuses and raising the possibility that this may also be a mechanism responsible for oral clefting in singletons.

Another scenario that may be related to hypoxia is high altitude. Data on birth defects in the highlands of South America have been systematically collected for more than three decades by the Latin American Collaborative Study of Congenital Malformations (ECLAMC). ECLAMC has operated since 1967 [58] and utilizes 70 hospitals and volunteer physicians in 12 countries in South and Central America to collect data on approximately 150,000 births per year (4 million since 1967). The birth prevalence of specific types of congenital anomalies at low and high altitudes in South America was compared after adjustment for prenatal growth, ethnicity, and socioeconomic status. The material includes all 1,668,722 consecutive births occurring in 53 hospitals participating in the ECLAMC, having registered at least 100 malformed/control pairs between 1967 and 1995. The lowland subsample (<6,500 ft above sea level) included 46,729 case-control pairs, ascertained in 1,539,432 births from 49 hospitals in 38 cities. The highlands (>6,500 ft) comprised 3,498 case-control pairs from 129,301 births, occurring in four hospitals from three cities (La Paz, Bolivia, Bogota, Colombia, and Quito, Ecuador). Unconditional logistic regression was used to estimate the relative risks for the exposure at high altitudes, adjusted by ethnicity, type of health service



and birth weight; showing significantly ($p < 0.01$) higher values in the high than in the lowlands for four types of defects: cleft lip, microtia, preauricular tag, branchial arch anomaly complex, constriction band complex and anal atresia. Conversely, lower risks in the highlands were registered for two neural tube defects (anencephaly and spina bifida) as well as for hydrocephaly and pes equinovarus. Even though some of these differences may be caused by undetected confounders, the coincidental finding of four types of craniofacial defects with higher, and two types of neural tube defect with lower frequencies in the highlands, suggests a real biological foundation [59].

Final Remarks

There is no doubt that cleft lip and palate is the birth defect in which we have learned the most about its etiology in the last two decades. The latest genome-wide association studies provided a new injection of excitement in the field, with the identification of never explored loci potentially playing a role in the defect, such as 8q24. These findings will be soon complemented by studies interrogating genome-wide coding sequences (whole exon-sequencing techniques) for mutations. These have the promise to unveil rare causal variants leading to cleft lip and palate that

will likely be related to single cases [3]. However, we now realize that the original hope of identifying genes that would fit major gene models of inheritance is not very likely. Another aspect that may affect our ability to associate genetic variation with susceptibility to cleft lip and palate (or any complex trait for that matter) is RNA editing. In this technique, enzymes target mRNA post-transcriptionally, deaminating adenosine to inosine, which in turn is recognized by the translation process as a guanosine (A to G), or changing cytidine to uridine (C to U) [60–66]. Additional evidence from mass spectrometry indeed suggests peptides can be translated from the discordant RNA sequences as well, and thus do not correspond exactly to the DNA sequences. This type of variation has not yet been explored but one can argue it could explain a portion of the defects and is part of the reason definitive results that can be translated to clinical practice from DNA variation association studies are yet to be obtained [67].

The redefinition of isolated cleft lip and palate using other clinical features appears to be a promising way to identify genes influencing the defect. We will stop using the terms ‘isolated’ or ‘non-syndromic’ in opposition of ‘syndromic’ forms of clefts and may start using more extensive clinical definitions, i.e. right cleft lip with cleft palate and bilateral lower second premolar and left upper lateral incisor agenesis. Another line of work still

Fig. 4. CDH1/Wnt pathways. There is evidence that genetic variation in *CDH1* and *AXIN2* is associated with unilateral right cleft of the lip and dental anomalies [50]. We also see more frequently the presence of dental anomalies opposite of the side of the clefted lip [46]. **a** During epithelial-mesenchymal transition, CDH1 (E-cadherin) in adherens junctions becomes phosphorylated, releasing the β -catenin bound to the cytoplasmic tail of E-cadherin into the cytoplasm. β -Catenin becomes part of a cytoplasmic complex that contains aside from β -catenin, the adenomatous polyposis coli protein (APC), glycogen-synthase kinase-3 β (GSK-3 β) and AXIN, and when it is phosphorylated, becomes ubiquitinated and is targeted for degradation. However, in response to a Wnt/frizzled signal, β -catenin is stabilized and transported into the nucleus, where it can participate with LEF1/TCF (lymphoid enhancer binding factor/T-cell factor) in the transcriptional induction of genes that are required for epithelial mesenchymal transition [76]. During dental development, *PAX9* (paired box 9) is upstream of *BMP4* (bone morphogenetic protein 4), which activates *MSX1* (muscle segment homeobox 1) and *LEF1* and subsequent transcription of other genes. *MSX1* also plays the role of inhibiting *BMP4* [77]. **b** As for the pathway including *nodal*, *lefty*, and *Pitx2* only found on the left side of the jaws [51], smaller amounts of β -catenin being released due to variation in *CDH1* (E-cadherin) or lesser amounts of β -catenin being complexed in the cytoplasm and later transported into the nucleus may increase the susceptibility to cleft of the lip in the right side and dental anomalies.

little explored is taking advantage of recently admixture populations. Certain parts of the world, such as the Americas, where different geographic groups were brought together over a relatively short period of time allow for the definition of more discrete groups based on population-specific genetic variation. Preliminary work under this assumption suggests more discrete groups can be defined using mitochondrial or Y chromosome variation that are over-represented among cases born with oral clefts [68, 69]. Also associations between candidate gene markers and isolated cleft lip and palate can be detected after analysis takes into consideration population-specific genotypes [10, 70].

The identification of environmental factors that increase or decrease susceptibility to oral clefts continues to be of great interest and study designs should incorporate this information. Unveiling the mechanism underlying maternal cigarette smoking and oral clefts would provide a new venue for dissecting possible pathways leading to the defect. Maternal cigarette smoking contributes to variation in DNA methylation of select CpG loci that can affect calculations of heritability. These variations include different methylation profiles between boys and girls, and are persistent years after birth [71]. Differentially variably

methyated regions in colon tumors and adjacent normal tissues show enrichment of genes regulating gene expression, cell morphogenesis, and development, supporting a biological role for DNA methylation variability in cancer [72]. Those relationships could also provide a rationale for the associations we see between cleft lip and palate occurrence and susceptibility to cancer.

We are moving into a new era of studies that will incorporate epigenetic variables, but we will need to first solve the issue of how information can be obtained and how these genetic variations can be assayed. It is likely that only DNA samples will not be enough to perform these kinds of studies, which allows us to predict that it will still be many years until meaningful new information that can really impact clinical care can be generated.

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References

- 1 Fraser FC: The genetics of cleft lip and palate. *Am J Hum Genet* 1970;22:336–352.
- 2 Ardinger HH, Buetow KH, Bell GI, Bardach J, VanDemark D, Murray JC: Association of genetic variation of the transforming growth factor- α gene and cleft lip and palate. *Am J Hum Genet* 1989;45:348–353.
- 3 Vieira AR: Unraveling human cleft lip and palate research. *J Dent Res* 2008;87:119–125.
- 4 Chung CS, Bixler D, Watanabe T, Koguchi H, Fogh-Andersen P: Segregation analysis of cleft lip with or without cleft palate: a comparison of Danish and Japanese data. *Am J Hum Genet* 1986;39:603–611.
- 5 Marazita ML, Spence MA, Melnick M: Major gene determination of liability to cleft lip with or without cleft palate: a multiracial view. *J Craniofac Genet Dev Biol* 1986;2:89–97.
- 6 Vieira AR: Association between the transforming growth factor- α gene and nonsyndromic oral clefts: a HuGE review. *Am J Epidemiol* 2006;163:790–810.
- 7 Zuccherro TM, Cooper ME, Maher BS, Daack-Hirsch S, Nepomuceno B, Ribeiro L, et al: Interferon regulatory factor 6 (*IRF6*) gene variants and the risk of isolated cleft lip or palate. *N Engl J Med* 2004;351:769–780.
- 8 Vieira AR, Goldstein McHenry T, Daack-Hirsch S, Murray JC, Marazita ML: A genome-wide linkage scan for cleft lip and palate and dental anomalies. *Am J Med Genet A* 2008;146:1406–1413.

- 9 Vieira AR, McHenry TG, Daack-Hirsch S, Murray JC, Marazita ML: Candidate gene/loci studies in cleft lip/palate and dental anomalies finds novel susceptibility genes for clefts. *Genet Med* 2008;10: 668–674.
- 10 Vieira AR, Cooper ME, Marazita ML, Orioli IM, Castilla EE: Interferon regulatory factor 6 (*IRF6*) is associated with oral-facial clefts in individuals that originate in South America. *Am J Med Genet A* 2007;143:2075–2078.
- 11 Prescott NJ, Lees MM, Winter RM, Malcolm S: Identification of susceptibility loci for nonsyndromic cleft lip with or without cleft palate in a two stage genome scan of affected sib-pairs. *Hum Genet* 2000;106:345–350.
- 12 Marazita ML, Field LL, Cooper ME, Tobias R, Maher BS, Peanchitlertkajorn S, et al: Genome scan for loci involved in cleft lip with or without cleft palate, in Chinese multiplex families. *Am J Hum Genet* 2002;71:349–364.
- 13 Zeiger JS, Hetmanski JB, Beaty TH, VanderKolk CA, Wyszynski DF, Bailey-Wilson JE, et al: Evidence for linkage of nonsyndromic cleft lip with or without cleft palate to a region on chromosome 2. *Eur J Hum Genet* 2003;11:835–839.
- 14 Wyszynski DF, Albach-Hejazi H, Aldirani M, Hammod M, Shkair H, Karam A, et al: A genome-wide scan for loci predisposing to non-syndromic cleft lip with or without cleft palate in two large Syrian families. *Am J Med Genet A* 2003;123:140–147.
- 15 Field LL, Ray AK, Cooper ME, Goldstein T, Shaw DF, Marazita ML: Genome scan for loci involved in nonsyndromic cleft lip with or without cleft palate in families from West Bengal, India. *Am J Med Genet A* 2004;130:265–271.
- 16 Marazita ML, Field LL, Tunçbilek G, Cooper ME, Goldstein T, Gürsu KG: Genome-scan for loci involved in cleft lip with or without cleft palate in consanguineous families from Turkey. *Am J Med Genet A* 2004;126:111–122.
- 17 Marazita ML, Murray JC, Lidral AC, Arcos-Burgos M, Cooper ME, Goldstein T, et al: Meta-analysis of 13 genome scans reveals multiple cleft lip/palate genes with novel loci on 9q21 and 2q32-35. *Am J Hum Genet* 2004;75:161–173.
- 18 Radhakrishna U, Ratnamala U, Gaines M, Beiraghi S, Hutchings D, Golla J, et al: Genome-wide scan for nonsyndromic cleft lip and palate in multigenerational Indian families reveals significant evidence of linkage at 13q33.1-34. *Am J Hum Genet* 2006;79:580–585.
- 19 Riley BM, Schultz RE, Cooper ME, Goldstein-McHenry T, Daack-Hirsch S, Lee KT, et al: A genome-wide linkage scan for cleft lip and cleft palate identifies a novel locus on 8p11-23. *Am J Med Genet A* 2007;143:846–852.
- 20 Marazita ML, Lidral AC, Murray JC, Field LL, Maher BS, Goldstein McHenry T, et al: Genome scan, fine-mapping, and candidate gene analysis of nonsyndromic cleft lip with or without cleft palate reveals phenotype-specific differences in linkage and association results. *Hum Hered* 2009;68:151–170.
- 21 Mangold E, Reutter H, Birnbaum S, Walier M, Mattheisen M, Henschke H, et al: Genome-wide linkage scan of nonsyndromic orofacial clefting in 91 families of central European origin. *Am J Med Genet A* 2009;149:2680–2694.
- 22 Wang Y, Li X, Zhu WL, Guo JZ, Song XM, Li SQ, et al: Genome-wide and interaction linkage scan for nonsyndromic cleft lip with or without cleft palate in two multiplex families in Shenyang, China. *Biomed Environ Sci* 2010;23: 363–370.
- 23 Birnbaum S, Ludwig KU, Reutter H, Herms S, Steffens M, Rubini M, et al: Key susceptibility locus for nonsyndromic cleft lip with or without cleft palate on chromosome 8q24. *Nat Genet* 2009; 41:473–477.
- 24 Grant SE, Wang K, Zhang H, Glaberson W, Annaiah K, Kim CE, et al: A genome-wide association study identifies a locus for nonsyndromic cleft lip with or without cleft palate on 8q24. *J Pediatr* 2009; 155:909–913.
- 25 Beaty TH, Murray JC, Marazita ML, Munger RG, Ruczinski I, Hetmanski JB, et al: A genome-wide association study of cleft lip with and without cleft palate identifies risk variants near MAFB and ABCA4. *Nat Genet* 2010;42:525–529.
- 26 Mangold E, Ludwig KU, Birnbaum S, Baluardo C, Ferrian M, Herms S, et al: Genome-wide association study identifies two susceptibility loci for nonsyndromic cleft lip with or without cleft palate. *Nat Genet* 2010;42:24–26.
- 27 Easton DF, Pooley KA, Dunning AM, Pharoah PD, Thompson D, Ballinger DG, et al: Genome-wide association study identifies novel breast cancer susceptibility loci. *Nature* 2007;447:1087–1093.
- 28 Hanson RL, Craig DW, Millis MP, Yeatts KA, Kobes S, Pearson JV, et al: Identification of PVT1 as a candidate gene for end-stage renal disease in type 2 diabetes using a pooling-based genome-wide single nucleotide polymorphism association study. *Diabetes* 2007;56:975–983.
- 29 Melzer D, Perry JR, Hernandez D, Corsi AM, Stevens K, Rafferty I, et al: A genome-wide association study identifies protein quantitative trait loci (pQTLs). *PLoS Genet* 2008;4:e1000072.
- 30 Thomas G, Jacobs KB, Yeager M, Kraft P, Wacholder S, Orr N, et al: Multiple loci identified in a genome-wide association study of prostate cancer. *Nat Genet* 2008;40:310–315.
- 31 Australia and New Zealand Multiple Sclerosis Genetics Consortium (ANZgene): Genome-wide association study identifies new multiple sclerosis susceptibility loci on chromosomes 12 and 20. *Nat Genet* 2009;41:824–828.
- 32 Eeles RA, Kote-Jarai Z, Al Olama AA, Giles GG, Guy M, Severi G, Muir K, et al: UK Genetic Prostate Cancer Study Collaborators/British Association of Urological Surgeons' Section of Oncology; UK ProtecT Study Collaborators; PRACTICAL Consortium, Easton DF: Identification of seven new prostate cancer susceptibility loci through a genome-wide association study. *Nat Genet* 2009;41: 1116–1121.
- 33 Shete S, Hosking FJ, Robertson LB, Dobbins SE, Sanson M, Malmer B, et al: Genome-wide association study identifies five susceptibility loci for glioma. *Nat Genet* 2009;41:899–904.
- 34 Dubois PC, Trynka G, Franke L, Hunt KA, Romanos J, Curtotti A, et al: Multiple common variants for celiac disease influencing immune gene expression. *Nat Genet* 2010;42:295–302.
- 35 Enciso-Mora V, Broderick P, Ma Y, Jarrett RF, Hjalgrim H, Hemminki K, et al: A genome-wide association study of Hodgkin's lymphoma identifies new susceptibility loci at 2p16.1 (REL), 8q24.21 and 10p14 (GATA3). *Nat Genet* 2010; 42:1126–1130.

- 36 Ferreira RC, Pan-Hammarström Q, Graham RR, Gateva V, Fontán G, Lee AT, et al: Association of IFIH1 and other autoimmunity risk alleles with selective IgA deficiency. *Nat Genet* 2010;42:777–780.
- 37 Franke A, McGovern DP, Barrett JC, Wang K, Radford-Smith GL, Ahmad T, et al: Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet* 2010;42:1118–1125.
- 38 Goode EL, Chenevix-Trench G, Song H, Ramus SJ, Notaridou M, Lawrenson K, et al: Wellcome Trust Case-Control Consortium, Beesley J, Webb PM; Australian Cancer Study (Ovarian Cancer); Australian Ovarian Cancer Study Group; Ovarian Cancer Association Consortium (OCAC), Chen X, Ekici AB, Thiel FC, Beckmann MW, Yang H, Wentzensen N, et al; Ovarian Cancer Association Consortium (OCAC): A genome-wide association study identifies susceptibility loci for ovarian cancer at 2q31 and 8q24. *Nat Genet* 2010;42:874–879.
- 39 Lango Allen H, Estrada K, Lettrec G, Berndt SI, Weedon MN, Rivadeneira F, et al: Hundreds of variants clustered in genomic loci and biological pathways affect human height. *Nature* 2010; 467:832–838.
- 40 Rothman N, Garcia-Closas M, Chatterjee N, Malats N, Wu X, Figueroa JD, et al: A multistage genome-wide association study of bladder cancer identifies multiple susceptibility loci. *Nat Genet* 2010;42:978–984.
- 41 Takata R, Akamatsu S, Kubo M, Takahashi A, Hosono N, Kawaguchi T, et al: Genome-wide association study identifies five new susceptibility loci for prostate cancer in the Japanese population. *Nat Genet* 2010;42:751–754.
- 42 Cui R, Okada Y, Jang SG, Ku JL, Park JG, Kamatani Y, et al: Common variant in 6q26-q27 is associated with distal colon cancer in an Asian population. *Gut* 2011;60:799–805.
- 43 Bille C, Winther JF, Bautz A, Murray JC, Olsen J, Christensen K: Cancer risk in persons with oral cleft – a population-based study of 8,093 cases. *Epidemiol* 2005;161:1047–1055.
- 44 Taioli E, Ragin C, Robertson L, Linkov F, Thurman NE, Vieira AR: Cleft lip and palate in family members of cancer survivors. *Cancer Invest* 2010;28:958–962.
- 45 Menezes R, Marazita ML, McHenry TG, Cooper ME, Bardi K, Brandon C, et al: AXIN2, orofacial clefts and positive family history for cancer. *J Am Dent Assoc* 2009;140:80–84.
- 46 Letra A, Menezes R, Granjeiro JM, Vieira AR: Defining cleft subphenotypes based on dental development. *J Dent Res* 2007;86:986–991.
- 47 Kuchler EC, Motta LG, Vieira AR, Granjeiro JM: Side of dental anomalies and taurodontism as potential clinical markers for cleft subphenotypes. *Cleft Palate Craniofac J* 2011;48:103–108.
- 48 Menezes R, Vieira AR: Dental anomalies as part of the cleft spectrum. *Cleft Palate Craniofac J* 2008;45:414–419.
- 49 Weinberg SM, Neiswanger K, Martin RA, Mooney MP, Kane AA, Wenger SL, et al: The Pittsburgh Oral-Facial Cleft Study: expanding the cleft phenotype. Background and justification. *Cleft Palate Craniofac J* 2006;43:7–20.
- 50 Letra A, Menezes R, Granjeiro JM, Vieira AR: AXIN2 and CDH1 polymorphisms, tooth agenesis, and oral clefts. *Birth Defects Res A Clin Mol Teratol* 2009; 85:169–173.
- 51 Boorman CJ, Shimeld SM: The evolution of left-right asymmetry in chordates. *Bioessays* 2002;24:1004–1011.
- 52 Beaty TH, Ruczinski I, Murray JC, Marazita ML, Munger RG, Hetmanski JB, et al: Evidence for gene-environment interaction in a genome-wide study of non-syndromic cleft palate. *Genet Epidemiol* 2011;35:469–478.
- 53 Boyles AL, DeRoo LA, Lie RT, Taylor JA, Jugessur A, Murray JC, et al: Maternal alcohol consumption, alcohol metabolism genes, and the risk of oral clefts: a population-based case-control study in Norway, 1996–2001. *Am J Epidemiol* 2010;172:924–931.
- 54 Wu T, Liang KY, Hetmanski JB, Ruczinski I, Fallin MD, Ingersoll RG, et al: Evidence of gene-environment interaction for the *IRF6* gene and maternal multivitamin supplementation in controlling the risk of cleft lip with/without cleft palate. *Hum Genet* 2010;128:401–410.
- 55 Wu J, Zheng Q, Huang YQ, Wang Y, Li S, Lu DW, et al: Significant evidence of association between polymorphisms in ZNF533, environmental factors, and nonsyndromic orofacial clefts in the Western Han Chinese population. *DNA Cell Biol* 2011;30:47–54.
- 56 Jones KL, Webster WS, Vaux KK, Benirschke K: Acardiac fetus: evidence in support of a vascular/hypoxia pathogenesis for isolated oral clefting. *Birth Defects Res A Clin Mol Teratol* 2008; 82:597–600.
- 57 Gimenez-Scherer JA, Davies BR: Malformations in acardiac twins are consistent with reversed blood flow: liver as a clue to their pathogenesis. *Pediatr Dev Pathol* 2003;6:520–530.
- 58 Castilla EE, Orioli IM: ECLAMC: the Latin-American collaborative study of congenital malformations. *Community Genet* 2004;7:76–94.
- 59 Castilla EE, López-Camelo JS, Campaña H: The altitude as a risk factor for congenital anomalies. *Am J Med Genet* 1999;86:9–14.
- 60 Chester A, Scott J, Anant S, Navaratnam N: RNA editing: cytidine to uridine conversion in apolipoprotein B mRNA. *Biochim Biophys Acta* 2000;1494:1–13.
- 61 Athanasiadis A, Rich A, Maas S: Widespread A-to-I RNA editing of Alu-containing mRNAs in the human transcriptome. *PLoS Biol* 2004;2:e391.
- 62 Levanon EY, Eisenberg E, Yelin R, Nemzer S, Halleger M, Shemesh R, et al: Systematic identification of abundant A-to-I editing sites in the human transcriptome. *Nat Biotechnol* 2004;22: 1001–1005.
- 63 Nishikura K: Editing the message from A to I. *Nat Biotechnol* 2004;22:962–963.
- 64 Conticello SG: The AID/APOBEC family of nucleic acid mutators. *Genome Biol* 2008;9:229.
- 65 Li JB, Levanon EY, Yoon JK, Aach J, Xie B, Leproust E, et al: Genome-wide identification of human RNA editing sites by parallel DNA capturing and sequencing. *Science* 2009;324:1210–1213.
- 66 Sakurai M, Yano T, Kawabata H, Ueda H, Suzuki T: Inosine cyanoethylation identifies A-to-I RNA editing sites in the human transcriptome. *Nat Chem Biol* 2010;6:733–740.
- 67 Li M, Wang IX, Li Y, Bruzel A, Richards AL, Toung JM, et al: Widespread RNA and DNA sequence differences in the human transcriptome. *Science* 2011; 333:53–58.
- 68 Vieira AR, Karras JC, Orioli IM, Castilla EE, Murray JC: Genetic origins of a South American clefting population. *Clin Genet* 2002;62:458–463.

- 69 Vieira AR, Pliss L, Pelnena I, Krumina A, Baumanis V, Luce B: Mitochondrial DNA origins of the Latvian clefting population. *Mitochondrion* 2011;11:357–359.
- 70 Vieira AR, Cooper ME, Marazita ML, Castilla EE, Orioli IM: Reduced folate carrier 1 (RFC1) is associated with cleft of the lip only. *Braz J Med Biol Res* 2008;41:689–693.
- 71 Breton CV, Salam MT, Gilliland FD: Heritability and role for the environment in DNA methylation in AXL receptor tyrosine kinase. *Epigenetics* 2011;6:895–898.
- 72 Jaffe AE, Feinberg AP, Irizarry RA, Leek JT: Significance analysis and statistical dissection of variably methylated regions. *Biostatistics* 2011;13:166–178.
- 73 Rahimov F, Marazita ML, Visel A, Cooper ME, Hitchler MJ, Rubini M, et al: Disruption of an AP-2α binding site in an *IRF6* enhancer is associated with cleft lip. *Nat Genet* 2008;40:1341–1347.
- 74 Paranaíba LM, Bufalino A, Martelli-Júnior H, Barros LM, Graner E, Coletta RD: Lack of association between *IRF6* polymorphisms (rs2235371 and rs642961) and non-syndromic cleft lip and/or palate in a Brazilian population. *Oral Dis* 2010;16:193–197.
- 75 Blanton SH, Burt A, Garcia E, Mulliken JB, Stal S, Hecht JT: Ethnic heterogeneity of *IRF6* AP-2α binding site promoter SNP association with nonsyndromic cleft lip and palate. *Cleft Palate Craniofac J* 2010;47:574–577.
- 76 Larsen M, Tremblay ML, Yamada KM: Phosphatases in cell-matrix adhesion and migration. *Nat Rev Mol Cell Biol* 2003;4:700–711.
- 77 Peters H, Neubuser A, Kratochwil K, Balling R: Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes Dev* 1998;12:2735–2747.

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The Mouse as a Developmental Model for Cleft Lip and Palate Research

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Abstract

Vertebrate and invertebrate model organisms are essential for deciphering biological processes. One of these, the mouse, proved to be a valuable model for understanding the etiopathogenesis of a vast array of human diseases, including congenital malformations such as orofacial clefting conditions. This small mammal's usefulness in cleft lip and palate research stems not only from the striking anatomical and molecular similarities of lip and palate development between human and mouse embryos, but also from its amenability to experimental and genetic manipulation. Using some recent studies as illustrative examples, this review describes different ways of generating and exploiting mouse models to study normal and abnormal development of the lip and palate. Despite a few surmountable disadvantages of using the mouse, numerous mutants have revealed a growing number of molecular key players and have pointed at a tight and complex molecular control during each step of lip and palate development.

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The mouse has transcended its humble role as a tiny part of the ecosystem to become a prominent player in the war against human ailments such as cancer, congenital malformations as well as neurological, metabolic, immunological, degenerative

and age-related diseases. While early embryologists and teratologists used the rat and the rabbit as mammalian models, these were overshadowed by the mouse following the advent of mouse genetics, the generation of inbred strains and the availability of scores of spontaneous, chemically and radiation-induced mouse mutants. Added to these advantages are the facts that mice have relatively short generation times, are prolific, small in size, and can be maintained in a cost-effective way. In the last decade of the 20th century, development of innumerable and increasingly innovative and powerful molecular and genetic tools enabling genetic manipulation of the mouse, combined later on with the completion of the mouse genome sequence, catapulted the use of the mouse in biomedical research to a new era. In parallel, mouse strain repositories and information resources, such as sequence, gene expression and phenotype databases, emerged and continue to facilitate the endeavor of scientific and industrial communities. In addition to mice with spontaneous mutations, thousands of mouse models of human diseases have been generated by different methods, including transgenesis, ethylnitrosourea (ENU)- or transposon-induced mutagenesis, gene-targeting and gene-trapping technologies. Work by international consortia is

underway with the challenging goal of disabling the 20,000 protein encoding genes in the mouse genome. Recently, a novel and powerful technology enabled, so far, the generation of more than 9,000 reporter (*lacZ*)-tagged conditional alleles in mouse embryonic stem cell lines, and is aiming at generating reporter-tagged conditional mutations in all murine protein-coding genes within the next few years [1].

In the field of cleft lip (CL) and cleft palate (CP) research, the mouse, in conjunction with human genetics studies, has been instrumental in providing insights into normal development of the lip and palate and unveiling clues about the genetic, molecular and cellular events that engender these frequent and devastating orofacial defects. Studies of transgenic and mutant mice have pointed at a bewildering number of molecular players during lip and palate development, including receptors, ion channels, signaling molecules, extracellular components, junctional complexes as well as cytoplasmic and nuclear effectors. Some elegant studies have demonstrated genetic interactions, as well as vertical and horizontal connections between signaling pathways. However, much work is still needed, and the challenge ahead is to understand how cells process the dazzling amount of signals they receive from within and from the neighborhood to reach the decision to remain unchanged, change their identity, proliferate, migrate, differentiate or die. Previous comprehensive reviews [2–7] have listed and described in detail the scores of mouse mutants and what they have taught us so far about normal development of the lip and palate as well as the etiopathogenesis of CL with and without CP (CL/CP) or clefting affecting only the secondary palate (CPO). This review will highlight the importance of the mouse as a model organism for CL and CP research, some of the clever genetic tricks used by researchers to bypass hurdles, and discuss experimental needs and the growing number of biological questions by describing a number of previously and newly generated mutants with CL, CL/CP or CPO.

The Mouse as a Model for Cleft Lip and Cleft Palate Research

Lip and palate clefting results from disruptions impacting on cellular migration, proliferation, programmed cell death (apoptosis), extracellular matrix deposition and/or morphogenetic movements. All of these developmental events are crucial during the formation of lip and palate primordia. In contrast to development of the upper lip and primary palate, development of the secondary palate takes place in conjunction with growth and differentiation of other structures in the head and oral cavity, including the craniofacial skeleton and tongue. Therefore, clefting of the secondary palate may be secondary to defects in these structures, especially if the gene is not expressed in the primordia of the palate proper [4, 5, 8]. However, CP can also be associated either with intrinsic disruptions within the palate proper or by a combination of both primary biological alterations within the palatal shelves and defects in other cranial structures [5, 9]. In view of the striking external differences between humans and mice, a non-specialist would question the usefulness of the mouse in CL and CP research. However, during early craniofacial development, mouse and the human embryos bear a striking resemblance and are essentially of similar sizes. In addition, development of the lip and palate are basically similar in the two species. More importantly and notwithstanding the fact that mice and humans share around 99% of their genes [5], numerous genes that have been incriminated as causal factors in human orofacial clefting also engender clefting in mice and vice versa [5].

CL and CP in humans is an end point and knowing what went wrong in the womb, even if the etiological factor has been identified, is impossible. However, mouse models allow us to track down the cellular and molecular events during the different steps of lip and palate development and flesh out the pathogenesis of a given clefting condition. In other words, using the

mouse embryo allows us to determine whether clefting is secondary to altered growth of lip or palatal primordia (due to altered cell proliferation, increased apoptosis, or abnormal migration of cranial neural crest cells which make up the bulk mesenchyme of these structures), lack of morphogenetic movements (failure of elevation of the palatal shelves or abnormal invagination of lip primordia), or abnormal fusion (due to perdurance of the transient epithelial seams as a result of abnormally sustained cell proliferation and/or lack of apoptosis, abnormal early adhesion between the epithelia of the abutting primordia that are fated to merge) [4, 5]. Whilst human genetics studies have been, to some extent, successful at identifying culprit genes in several syndromic forms of orofacial clefting, deciphering the etiology of non-syndromic forms of CL and/or CP is a more challenging task, in view of their complex and multifactorial etiology. A gene could be considered as a good candidate for non-syndromic clefting based on its chromosomal location, if it is expressed during the critical steps of lip and palate development, and/or when its dysfunction generates CL and/or CP in mice. Molecular analyses of mutant mice and gene expression profiles in developing murine lip and palate primordia also enable scientists to unveil regulatory networks and to identify new candidate genes that may be involved in human clefting [4, 5].

Biochemical and Genetic Tools

In addition to the availability of different methods for the generation of mice, which model an array of human diseases, including congenital malformations, complementary techniques and tools for mouse studies are abundant and are becoming more and more sophisticated. In developmental biology, methods such as whole-mount staining of embryos or organ primordia for the visualization of transcripts, small molecules, and endogenous or reporter proteins are particularly

informative. Similarly, skeletal preparations with alcian blue and alizarin red staining provide clues about the extent of defects in craniofacial skeletal elements. These can be combined with 3-D reconstructions, histological sections and tissue extracts at different developmental stages, as well as the use of genetically-modified cell lines and organ culture, combined with immunological and molecular analyses to generate a wealth of information. Organ culture of palatal shelves from mouse embryos provide clear answers to determine whether a gene product is important for palatal fusion, and this methodology has been used in numerous studies [4, 10, 11]. This in vitro system has also been elegantly used to knock-down gene expression and to test the biological effects of signaling molecules as well as small molecule inhibitors [4, 5, 11, 12]. As stated above, there exists a vast array of methods for the generation of mutant mice and the reader is referred to excellent chapters describing the principles and technical aspects of these methods [13, 14].

Mutants Generated by Gene Targeting

Gene-targeting methods are based on DNA homologous recombination in mouse embryonic stem (ES) cells. These strategies modify genes in their original location in the genome and allow to either remove an important part of the gene (knock-out) or to insert a reporter gene (or a recombinase gene, or both) into an endogenous locus under the control of an endogenous promoter (knock-in). The spatiotemporal expression of the knocked-in reporter replicates that of the endogenous gene, thus providing a direct readout for the expression of the targeted gene. Gene targeting in ES cells or in the epiblast can also be used to generate allelic mutations in a given gene to produce mice harboring gain-of-function or partial loss-of-function, as well as mice bearing mutations that replicate those in humans. Gene targeting in ES cells is further refined to produce conditional alleles which, in conjunction with a site-specific recombination system, lead to inactivation of the

targeted gene by recombination in tissue-specific and temporally controlled manners.

Ubiquitous Gene Alterations

Ubiquitous genetic alterations entails that homozygous null mutants or 'knock-outs' as well as 'knock-ins' display loss-of-function or altered function (for example by introduction of point mutations) of a given gene in all cells of the body, regardless of the strategy used to remove or modify the gene. Some mutants exhibit a clefting phenotype in the heterozygous state. This is well illustrated in heterozygous mutants for *Tcof1*, *Satb2* and *Sumo1*, encoding the nucleolar protein Treacle, the transcription factor Satb2, and the small ubiquitin-related modifier 1, respectively [5]. However, most clefting phenotypes in mice are recessive, occurring only upon ablation of the function of both alleles of a given gene [5]. Numerous mouse models with ubiquitous gene alteration show CL/CP or CP, some of which have provided invaluable information about the gene of interest and its protein product, including expression patterns, function, regulation, targets and location in molecular networks, during normal and abnormal lip and palate development [2–7].

Clefting Caused by Altered Growth of Lip and Palate Primordia. A gene identified as disrupted in humans suffering from brain tumors would have long remained unsuspected as part of the molecular activity underlying palatogenesis. However, null mutants for *Meningioma1* (*Mn1*^{-/-}), encoding a transcription factor, were found to exhibit CP, primarily caused by altered growth of the middle and posterior segments of the palate [9]. Interestingly, *Mn1* transcripts are to a large extent co-expressed with *Tbx22*, and *Mn1*^{-/-} mutants displayed reduced expression levels of *Tbx22*. In humans, *TBX22* mutations cause both syndromic (X-linked CP and ankyloglossia, CPX) and non-syndromic CP [5]. Recently, *Tbx22* null mice were found to exhibit ankyloglossia and a submucous CP (SMCP), replicating the situation in some patients with CPX [15]. The SMCP was caused by

ossification defects in the posterior portion of the hard palate, the domain of expression of *Tbx22*. Reporter assays in cell culture revealed that Mn1 acts as a transcriptional activator of *Tbx22* expression [9]. These findings are important as they put the two transcription factors in the same molecular pathway and constitute a basis for further work to decipher their mode of action and regulation.

Signaling by Eph receptors and their ligands, ephrins, is involved in the regulation of a vast array of cellular processes, including cell-extracellular matrix adhesion, cell-cell contacts, cell-sorting and aggregation phenomena, as well as in controlling cell shape, migration, proliferation, differentiation, and secretion [16]. This signaling pathway is intriguing because both the receptors (forward signaling) and the ligands (reverse signaling) are able to transduce signals subsequent to interaction with each other [16, 17]. Mice in which both the *EphB2* and the *EphB3* genes, encoding high-affinity ephrin-B1 receptors, are disabled (*EphB2*; *EphB3* compound mutants) exhibit CP, an abnormal corpus callosum and defectuous axon tracts [18]. However, this model is not useful to distinguish between forward and reverse signaling. EphB receptors and ephrin-B1 ligands are expressed in the palatal shelves (PS), suggesting endogenous function of this signaling pathway during palatogenesis [18–20]. In humans, mutations of the gene encoding ephrin-B1 cause craniofrontonasal syndrome, leading to developmental anomalies of the nervous system and skeletal and craniofacial malformations, including CL and CP. Furthermore, in humans, *EPHB2* mutations have been incriminated in craniofacial malformations, including orofacial clefting [19 and references therein]. Although these facts suggest a role of EphB-ephrin signaling during palatogenesis, clues from mouse models only recently began to emerge providing a better understanding of the function of this signaling pathway in the developing palate.

Genetic ablation of *ephrin-B1* specifically in the neural crest using the *Cre/loxP* system (see Tissue-

Specific Gene Targeting) recapitulated to a large extent the palatal clefting phenotype seen in *ephrin-B1* null mutants [17]. Whether the clefting results from ephrin-B1 dysfunction within the palate proper or whether the phenotype is a mere consequence of early disruption of neural crest cell biology remained unclear. However, recent insightful studies revealed the importance of endogenous EphB-ephrin forward signaling for normal palate growth using different approaches [19–21]. Mice harboring mutations that disable ephrin-B1 reverse signaling are exempt from craniofacial and skeletal malformations, rather they display defects in axon guidance [21], a phenotype distinct from that of ephrin-B1 null and heterozygous mutants, which suffer from craniofacial malformations, including CP [17, 22]. This indicates an involvement of ephrin-B1 forward signaling during palate development. Furthermore, compound mutants (*EphB2^{lacZ/lacZ}*; *EphB3^{-/-}*) harboring null alleles for *EphB3* and a mutant *EphB2* gene, the product of which is unable to participate in forward signaling (its cytoplasmic domain-coding region was replaced by the *lacZ* gene) but can still activate reverse signaling, exhibit CP [20]. The CP in the *EphB2^{lacZ/lacZ}*; *EphB3^{-/-}* compound [20] and the *ephrin-B1* null and heterozygous [19] mutants was found to be caused by reduced size of the PS owing to diminished mesenchymal cell proliferation, but the PS were able to elevate [19, 20] and fuse when forced into contact in vitro [20]. In contrast to other molecules operating during palatogenesis, which when disrupted generate a minor palatal clefting confined to their expression domains [4, 5], ephrin-B1 dysfunction causes a complete cleft of the palate along its anterior-posterior axis, despite its expression being confined to the anterior palate and the fact that only the anterior palate is growth-retarded [19]. This generalized palatal clefting phenotype could be a result of disruption of intrinsic ephrin-B1 function within the anterior palate combined with other craniofacial malformations. This issue could be clarified by the removal of ephrin-B1 function, specifically in

the anterior palate. Craniofrontonasal syndrome is X-linked, however heterozygous female patients seem to exhibit more severe anomalies than males [19 and references therein]. This situation is in fact recapitulated in mice, since heterozygous *ephrin-B1^{+/-}* mice display more severe anomalies, including smaller PS than *ephrin-B1^{Y/-}* hemizygous or even *ephrin-B1^{-/-}* homozygous mutants [17, 19, 22]. These features have been suggested to be due to random X inactivation leading to mosaicism in tissues, i.e. some cells retain ephrin-B1 function, while others lose it [17, 19, 22]. In the case of the palate and telencephalon, the proportion of ephrin-B1-negative cells was shown to be outgrown by that of ephrin-B1-positive cells as a consequence of the relatively reduced proliferation rates of the mutant cells. Within the mosaic PS of *ephrin-B1^{+/-}* mutants, an altered distribution of proliferating cells could thus be the cause for the PS being more affected than in the *ephrin-B1* null mutants [19]. Analysis of *ephrin-B1* null and heterozygous mosaic PS revealed an unexpected upregulation of EphB3 and, to a lesser extent, EphB2 receptor proteins in ephrin-B1-negative cells, a phenomenon that was suggested to be due to altered post-transcriptional events leading to failure of endocytosis and subsequent degradation of EphB3 proteins in the absence of ephrin-B1 [19]. Interestingly, ephrin-B1 forward signaling was found to regulate cell proliferation in the palatal cells through the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signal transduction cascade [19]. However, the molecular intermediates linking the two signaling pathways remain to be determined. Nevertheless, activated ERK complex operates in the developing palate [23], and ERK signaling has been recently shown to be crucial for craniofacial and palate development with the use of conventional *ERK1* and neural-crest-specific *ERK2* ablations [24]. The *Mn1*, *EphB2/EphB3* and *ephrin-B1* mutants join a growing number of previously [4, 5] and newly [12, 25] generated models stressing the importance of PS growth for normal palatogenesis.

Altered cell migration within the orofacial primordia or intrinsic defects in proliferation and/or apoptosis can also lead to orofacial clefting. p63 is a transcription factor expressed in epithelia of orofacial primordia. Mutations in *P63* cause syndromic and non-syndromic CL/CP in humans [5], and *Tp63* null mutant mice display bilateral CL and CP [26]. The lip clefting in *Tp63* mutants was recently found to be caused by increased epithelial apoptosis owing to overproduction of bone morphogenetic protein 4 (BMP4), a signaling molecule known to be required for physiological apoptosis in some developmental settings. As a consequence, mitogenic stimuli from epithelium to mesenchyme of the lip primordia were defective, resulting in truncated lip primordia that were unable to merge [26]. A CL phenotype due to altered cell proliferation was recently found to occur in mice compound null homozygous for *Pax9* and *Msx1*, demonstrating interactions between the two genes [27]. *Msx1* and *Pax9* are notorious key players during craniofacial, palate and tooth development in humans and mice [5].

The knock-in strategy can be implemented to study functional differences and similarities of paralogous factors. This is well illustrated by mutants in which the coding sequence of the gene encoding the transcription factor Odd-skipped related 2 (*Osr2*) was substituted by that of *Osr1* or the A isoform of *Osr2* (*Osr2A*) [28]. That study was undertaken to determine the reasons behind the distinct malformations encountered in *Osr1* and *Osr2* null mutant mice, with the former exhibiting heart and urogenital defects, while the latter, born with open eyelids, succumb as a result of craniofacial anomalies, including CP caused by altered growth and morphogenesis [28, 29 and references therein]. Interestingly, both the knock-in alleles rescued the CP and craniofacial malformations in *Osr2* null mice. Thus, the different phenotypes of *Osr1* and *Osr2* mutants are not due to differences in the activities of the proteins, but rather due to the action of distinct *cis*-regulatory elements eliciting different spatiotemporal expression patterns [28].

Clefting Caused by Altered PS Elevation. A slight delay in PS elevation or diminished extension of the elevated PS engenders CP. Although the exact mechanisms triggering PS elevation are to date unclear [5], several mouse models revealed molecular factors that are crucial for PS elevation [5]. Studies of *Wnt5a* mutants with CP disclosed intriguing dual opposing roles for *Wnt5a* in regulating cell proliferation and gene expression along the anterior-posterior palatal axis and in controlling differential cell migration within the palate. These properties have been shown to be crucial for PS growth and elevation [12]. Other interesting mouse models revealed novel molecular players in PS elevation and their subsequent growth. These include mutants for the *Zfmx1a* [30] and *Vlk* [31] genes as well as a series of allelic compound mutants for genes encoding members of the ADAMTS (disintegrin-like and metalloproteinase domain with thrombospondin type 1 motif) family and the proteoglycan versican [32]. *Vlk* (*Vertebrate lonesome kinase*) encodes a novel protein kinase regulating the rate of transport of proteins from the Golgi apparatus to the plasma membrane. However, the *modus operandi* of its kinase in the Golgi is yet to be characterized [31]. Intriguingly, despite its high conservation among vertebrates, *Vlk* lacks invertebrate homologs [31]. *Vlk*^{-/-} mice develop skeletal anomalies resulting from delayed endochondral ossification and die shortly after birth from CP and lung defects [31]. In the developing palate *Vlk* is differentially expressed, with the highest expression levels being confined to the mesenchyme of the medial half of the middle palatal segment [31]. Analyses of *Vlk*^{-/-} mutants disclosed a complete failure of PS elevation in some embryos, whereas others displayed delayed PS elevation. However, no alterations in cell proliferation and apoptosis were noted in the PS [31]. Several molecular players during palatogenesis have been shown to exhibit differential expression patterns along the A-P and medial-lateral axes of the palate [4, 5, 33]. These may translate into differential cell proliferation

and extracellular matrix deposition necessary for the morphogenetic movements leading to PS elevation. In view of the importance of proteoglycans (PG) in PS elevation [4, 5, 32] and the localization of *Vlk* gene product in the Golgi [31], site of phosphorylation of PG [34], a link between *Vlk* protein and PG may exist [31]. The phenotype of the *Vlk*^{-/-} mutants, including the CP, was found to strikingly phenocopy that of *Fgf18* mutants, as well as that of mice with a spontaneous mutation of the gene encoding aggrecan, suggesting functions for *Vlk* in quantitatively regulating matrix proteins [31]. The role of the extracellular matrix in PS elevation [4, 5] and further growth of the elevated PS was further emphasized in compound mutants for the *Adamts20* and *Adamts9* genes. These encode a subgroup of secreted metalloproteinases of the ADAMTS family, known as proteoglycanases due to their ability to process large PG such as aggrecan and versican [32, 35], the latter being expressed in the developing palate [32, 36]. The *Adamts20* mutation has been identified in the spontaneous mutants *belted* (*bt*), whereas the *Adamts9* mutants are knock-ins. The homozygous *Adamts9* mutant embryos die at embryonic day 7.5 (E7.5), whereas *bt* homozygous mutants display pelage hair pigmentation defects and are viable, though a closer scrutiny revealed that some 3% of these display CP and thus would be non-viable [32]. Interestingly, *Adamts9*^{+/-}; *bt/bt* double mutants were found to develop a fully penetrant CP, whereas mutants with allelic combinations between these genes and *Adamts4* or *Adamts5* were viable, indicating specific functional relationships between ADAMTS9 and ADAMTS20 [32]. The CP in the *Adamts9*^{+/-}; *bt/bt* compound mutants was found to be secondary to delayed PS elevation as well as to decreased cell proliferation and cell density of the elevated PS, which otherwise were able to fuse in organ cultures. Furthermore, observations of the developing palate in other allelic series of *Adamts9* and *bt* revealed that ADAMTS9 and ADAMTS20 have independent functions during its development,

consistent with their expression domains, with *Adamts9* expression being confined to endothelia of capillaries, and *Adamts20* transcripts being broadly distributed in the PS mesenchyme. Interestingly, the processing of versican was found to be reduced in the palatal mesenchyme, but not around capillaries in the PS of the *Adamts9*^{+/-}; *bt/bt* mutants [32]. Moreover, *Adamts20* and *Vcan*, encoding versican were found to interact genetically inasmuch as compound *bt/bt*; *Vcan*^{hdf/+} mutants exhibited higher frequency of CP than the *bt/bt* mutants. The *bt/bt*; *Vcan*^{hdf/+} also displayed reduced clearance of versican as well as cell proliferation defects [32]. The recessive lethal heart defect (*hdf*) mutations arose in a transgenic line following insertion of a *lacZ* construct into the mouse genome, and the *Vcan* gene has been identified as disrupted by this insertion [37]. This elegant study re-emphasizes the role of versican as not just a space filler, but also as a regulator of cell proliferation and cellular density necessary for growth and morphogenesis.

Clefting Caused by Failure of Fusion of Lip and Palate Primordia. Mouse models have underpinned the importance of the adhesion and subsequent fusion of primordia of the lip and palate [2–5]. The medial epithelial seam (MES) that develops upon adhesion of the palatal medial edge epithelia (MEE) must degenerate progressively yet rapidly to allow mesenchymal continuity and successful palatal fusion. In several mouse models, altered apoptosis of the MES is the salient cause of failure of PS fusion [2, 4, 5]. Several factors have been shown to be required for palatal fusion, however to date the mechanisms by which transforming growth factor- β (TGF- β) signaling regulates this process is the one best understood [2, 4, 5].

Mutations in the gene encoding the transcription factor *Foxe1* cause syndromic (Bamforth-Lazarus syndrome) CP and are implicated in non-syndromic clefting in humans [5]. Despite the availability of *Foxe1* null mutant mice, which display CP, the role of this factor during palatogenesis

remained unclear. The PS of *Foxe1* mutants elevate but are unable to fuse [5]. Recently, investigations combining molecular assays in cell cultures and analyses of *Foxe1* null embryos revealed *Tgfb3* and *Msx1* encoding TGF- β 3 and the transcription factor *Msx1*, respectively as direct targets and positively regulated by *Foxe1* [38]. Given the crucial role of TGF- β 3 in apoptosis-dependent palatal fusion, this finding provides the first mechanistic glimpse of the function of *Foxe1* during palate fusion.

The use of compound mutants for genes belonging to same or different families is an invaluable means to disentangle phenomena due to functional redundancy and to uncover genetic interactions [5]. Pertaining to this, a couple of recent studies [10, 39] used compound heterozygous mutant mice for a knock-in mutation in *Interferon regulatory factor 6* (*Irf6*) and ablation of *Tp63*, together with complementary analyses in cell cultures, skin specimens from affected patients as well as homozygous mice, to reveal genetic interactions between *Irf6* and *p63* during palate development. *Irf6* encodes a transcription factor required for normal lip and palate development in both humans and mice. In humans, mutations of *IRF6* cause van der Woude (VWS) and popliteal pterygium (PPS) syndromes and are involved in non-syndromic orofacial clefting [5, 40]. The studies [10, 39] also revealed that these factors function in a regulatory loop to regulate epithelial proliferation and differentiation in the developing palate, wherein *p63* directly regulates expression of *Irf6*, thereafter IRF6 targets *p63* to degradation. Given the crowded conditions in the oral cavity of the developing embryo, it is a miracle that epithelia of structures such as the tongue, PS and other oral structures do not adhere to one another, yet the fusion of the palate requires adhesion of the MEE of the bilateral PS. Interestingly, humans and mice with *IRF6/Irf6* mutations as well as mice mutants for genes encoding fibroblast growth factor 10 (FGF10), or a ligand for the notch receptors, *Jagged2* (*Jag2*) [5 and references

therein] display intraoral adhesions, preventing the PS from elevating. Information about the molecular and cellular defects leading to such abnormal adhesions and the mechanisms required for palatal fusion has been provided through studies of mutants with mutations in *Irf6* as well as compound mutants for *Irf6* and *Tp63* [5 and references therein, 9]. In addition, compound heterozygotes for mutations in both *Irf6* and *Jag2* revealed that these factors function within a convergent molecular pathway to regulate formation and differentiation of an embryonic cell layer, the periderm, necessary for adhesion and fusion of the PS at the right places [41].

Functional redundancy of gene function during palatal fusion is well portrayed upon genetic inactivation of *Snai1* and *Snai2*, encoding the transcription factors Snail and Slug, respectively. Loss of *Snai2* in mice generates an incompletely penetrant CP, whereas removal of one *Snai1* allele in *Snai2* homozygotes (*Snai1*^{+/-}; *Snai2*^{-/-}) renders the CP fully penetrant [42].

Compound homozygous mutants with loss-of-function of both α v β 6 and α v β 8 integrins (*Itgb6*^{-/-}; *Itgb8*^{-/-}) recapitulate the vascular anomalies and the CP caused by loss of TGF- β 1 and TGF- β 3, respectively [43]. Mutants lacking the function of all α v integrins have a CP due to failure of PS fusion [5]. Furthermore, and in view of the ability of α v β 6 and α v β 8 integrins to activate both TGF- β 1 and TGF- β 3 through interactions with an RGD sequence within the latent forms of TGF- β 1 and TGF- β 3 [43 and references therein], the CP in *Itgb6*^{-/-}; *Itgb8*^{-/-} mutants is likely a result of defectuous palatal fusion. The CP in *Tgfb3* null mice as well as in other mutants with disrupted TGF- β signaling is secondary to failure of fusion of the PS caused by defectuous adhesion of the MEE and/or inability of the MES to disintegrate by apoptosis [2, 5, 7]. In addition to known targets and effectors of TGF- β signaling during palatal fusion [2, 5, 7], work in the mouse points at a role in palate fusion for β ig-h3 [44], a TGF- β -induced extracellular protein [45] shown to be expressed

in anatomical structures undergoing apoptosis, including in the MES [44, 45].

Altered function of developmental factors can cause not only congenital malformations but benign and malignant tumors as well. This is exemplified by mutations in genes encoding components of the BMP, Wnt and Hedgehog signaling pathways [5, 9]. Mutations in the gene encoding the tumor suppressor FBXW7, a substrate recognition element of an SCF-type E3 ubiquitin ligase complex, are found in several human cancers [46 and references therein]. Null mutant mice for *Fbxw7* die of vascular anomalies during early embryogenesis. However, unlike heterozygotes for an *Fbxw7* null allele (*Fbxw7*^{+/-}), which are viable and exempt of malformations, heterozygotes carrying a common point mutation (*Fbxw7*^{+/R482Q}) in the substrate recognition domain of FBXW7 die soon after birth and exhibit lung defects, CP and open eyelids but no gross craniofacial anomalies [46]. Thus, the anomalies in the *Fbxw7*^{+/R482Q} heterozygotes are not secondary to *Fbxw7* haploinsufficiency. It seems (from figure 4 in Davis et al. [46]) that, at least in the anterior region of the palate, the mutant PS elevated but did not fuse. Strikingly, the open eyelids, lung and CP phenotypes are reminiscent of *Jeff* mutants harboring a point mutation in the *Fbxo11* gene (*Jeff* mutants were identified following a phenotype-driven mutagenesis screen) encoding another component of the SCF-type E3 ubiquitin ligase complex. In these mutants the PS elevate on schedule but fail to fuse [47]. Importantly, the lung anomalies and CP without any other major craniofacial deformations as displayed by the *Jeff* and *Fbxw7* mutants are reminiscent of those in *Tgfb3* null mutants. *Fbxo11* and *Smad2*, encoding an effector in the TGF- β signal transduction cascade, seem to interact genetically since in contrast to heterozygotes for either gene, double heterozygotes for both genes (*Jeff*^{+/-}; *Smad2*^{+/-}) develop CP [47]. The levels of phosphorylated SMAD2 protein were found to be increased in palatal specimens of *Jeff* homozygotes, however no physical interactions

between SMAD2 and FBXO11 proteins were detected [47]. The substrates within the palate of the protein products of *Fbxo11* and *Fbxw7* genes are yet to be identified, and the molecular events leading to fusion defects in the mutants are at present unclear. The use of in vivo investigations combined with PS cultures would give the first glimpse about the mechanisms involved, as it is difficult to obtain a clear view of the biological events involved when comparing molecular marker expression patterns or levels between the MES of fused PS in control embryos with those of MEE of unfused PS of mutants.

As we have learned from mouse models for various diseases, too much or too restricted apoptosis is deleterious for embryogenesis and tissue homeostasis. The recently generated allelic mutations in compound mutants for genes encoding a subgroup of tyrosine phosphatases, the leukocyte antigen-related (LAR) receptor protein tyrosine phosphatase subfamily, known to reversibly control phosphorylated tyrosine-dependent activities, provide further understanding of the regulation of apoptosis during embryogenesis. In this regard, mice of the genotype *Ptprs*^{-/-}; *Ptprf* ^{$\Delta f/\Delta f$} , which totally lack the protein product of *Ptprs* and also harbor mutant *Ptprf* alleles (*Ptprf* ^{Δf}) resulting in a protein lacking its phosphatase activity, show various defects. These include craniofacial malformations (CP, exencephaly, micrognathia, abnormal tongue, as well as eye defects such as persistence of the hyaloid arteries, retinal hyperplasia and open eyelids at birth) and malformations of the urinary tract. Some of these anomalies were recapitulated, though at a lower frequency than in the compound homozygotes, in compound mutants carrying allelic series of these genes [48]. A salient feature of the urinary tract anomalies was found to be caused by persistence of the common nephric duct as a result of diminished caspase 8-independent (intrinsic) apoptosis [48]. The PS of the compound homozygotes elevated and seemed to have attained optimal growth, yet they did not fuse. In view of the known role of

apoptosis in elimination of the hyaloid artery and palate fusion, together with the findings in this study [5, 48, 49], it is very likely that altered apoptosis was the cause of CP in these mutants.

Alternatively, sustained epithelial proliferation of the MEE can also cause palatal clefting, as shown in mice conditionally lacking the function of TGF- β RII in the palatal epithelium [50]. Another possible function of these LAR subfamily members during palatogenesis may pertain to their role in maintaining cadherin complex stability [51] and, thus, regulating MEE adhesion. However, and keeping in mind their micrognathia, it remains to be determined whether the CP in the above *Ptprs*^{-/-}; *Ptprf* ^{$\Delta f/\Delta f$} mutants is due to intrinsic defects within the PS, if these tyrosine phosphatases indeed turn out to be operative within the palate, is a result of craniofacial malformations, or is caused by both phenomena.

Mutants Generated by Other Strategies

Mutants can be generated by chemical- or radiation-induced mutagenesis, random insertional mutagenesis or by transgenic means. Transgenic mice can be engineered by different methods to express a foreign gene. The transgene can be designed so that it is expressed either under heterologous or endogenous regulatory elements in a specific tissue, and it can be drug-inducible. The latter strategy allows the transgene to be turned on at a chosen time during development. Depending on the inducible system chosen, drugs such as tamoxifen or RU-486 are used. The tetracycline-inducible gene expression system is versatile as it allows the transgene to be turned on or off at will [13, 14]. Insertional mutagenesis uses viruses, transposons or gene trap strategies [13, 14, 52, 53].

Tissue-Specific Gene Targeting

While disrupting a gene of interest in the entire organism has been insightful in studies of normal and abnormal lip and palate development, it can lead to frustration if the mutant embryos die at early stages of development before initiation or

completion of lip and palate development. Equally frustrating are mutations that generate severe craniofacial malformations, leading to absence of orofacial structures [5]. There are however clever tricks to overcome these hurdles.

One of them is the ability to disable a gene or to introduce a genetic activating mutation in the tissue of choice and at a chosen developmental time [13, 14, 54]. The most popular system for this 'conditional gene targeting' approach uses the *Cre-loxP* system. This strategy can also be used to ubiquitously modify genes by using ubiquitous Cre deleter mice. The *Cre-loxP* system is a binary system that uses crosses between transgenic or knock-in mouse lines (usually males) that express the site-specific Cre recombinase in specific tissues with mice carrying *loxP*-flanked ('floxed') critical sequences of the targeted gene that are important for protein function. In the mutants, cells and tissues that carry both the *Cre* gene and the floxed allele undergo recombination events that lead to disruption of the floxed allele. The spatiotemporal distribution of Cre activity can be monitored by crossing *Cre*-expressing mice with animals harboring a reporter gene (encoding an enzyme such as β -galactosidase or a fluorescent protein). The *Cre-loxP* system can also be exploited in genetic fate mapping studies of normal and mutant cells [13, 14, 54]. An impressive number of mutants generated by *Cre-loxP* and harboring conditionally deactivated genes in neural crest cells during early embryogenesis as well as in the developing lip and palate epithelia, mesenchyme, or in both have been of great value in CL and CP research [5 and references therein]. Compound mutants harboring conditionally disabled genes together with ubiquitous null mutations for paralogs [24] or other genes [55], mutants overexpressing genes at orthotopic sites [56] or mice with genetic misexpression at ectopic sites [57] provided knowledge about functional redundancy, gene dosage effects and genetic interactions within a specific tissue. More recently, tissue-specific mutants for components of the TGF- β [11, 58], BMP [59, 60],

Wnt [55, 61], FGF [56, 62] and Hedgehog [63, 64] signaling pathways, as well conditional mutants with disabled functions of Hand2 and glycogen synthase kinase 3 β [65, 66], have uncovered previously unsuspected biological roles for these genes during the different steps of lip [55, 58] and palate [11, 59–66] development.

During development, a given gene product can function during every step of ontogenesis, although its function, modulated by other cell-autonomous and non-cell-autonomous factors can elicit different cellular responses such as cell fate changes, proliferation, apoptosis and differentiation. Other genes function only transiently in precursor cells, thereafter their expression is shut off as cells differentiate. Persistent function of these genes can thus generate disease. In this context, the *Cre-loxP* system combined with the knock-in strategy has been exploited to show that persistent expression in the palate (E13.5) of *Pax3*, encoding a transcription factor, leads to CP owing to impaired palatal mesenchymal cell differentiation and osteogenesis. Furthermore, *Pax3* was shown to directly regulate the expression of *Sosdc1* encoding a BMP inhibitor, making *Pax3*-expressing cells insensitive to BMP-induced bone formation [57]. Indeed, BMP signaling in the palate is required not only for growth, but also for osteogenesis, since ablation of *Bmpr1a* specifically in the palatal mesenchyme generates an anteriorly localized CP due to diminished cell proliferation, whereas the rest of the secondary palate displays a submucous cleft caused by failure of bone formation in the palatal processes of the maxilla [60]. On the other hand, conditional ablation of *Bmp7* in the germline leads to clefting of the soft palate, the posterior extension of the secondary palate containing muscles [67]. Accumulating evidence in mice [5, 59, 60, 67] suggests a role for BMP signaling during lip and palate development, and recent genetic studies point at the involvement of mutations in *BMP4* and *BMP7* genes in syndromic clefting [68, 69] and mutations in the *BMP4* gene in non-syndromic clefting [70]. In the latter,

patients with missense and nonsense mutations in *BMP4* were found to display either overt CL, subepithelial alterations in the muscle orbicularis oris, or ‘microform cleft lip’, i.e. subtle defects that indicate that either lip and alveolar ridge development was delayed or that healing took place in the fetus [70]. Remarkably, healing of CL has been reported to occur in mice with conditional loss of function of *Bmp4* in lip primordia [71]. On another note, the role of the neurotransmitter γ -aminobutyric acid (GABA) in the developing palate and the pathogenesis of CP upon disruption of GABA signaling have been subject to discordances [5]. Findings from experimental manipulation and in mutants lacking *Gad1*, the gene encoding a GABA-synthesizing enzyme, mutants for a subunit in the GABA_A receptor (*Gabrb3*) as well as in *Viaat* mutants lacking the function of a vesicular carrier for both GABA and glycine, indicate that the CP is due to failure of or delayed PS elevation. However, whether this is caused by intrinsic defects within the palate or to altered fetal movements subsequent to dysfunction of the central nervous system (CNS) remained unclear [5]. Using different experimental approaches as well as CNS-specific inactivation of *Gad1*, a recent study [72] demonstrated that disruption of GABA signaling solely in the CNS indeed led to CP owing to altered fetal motor activity. Altered fetal movement (akinesia) has been incriminated in malformations of several organs and tissues [72 and references therein], and these may as well be causes of non-syndromic clefts in humans with mutations in the *GAD1* and *GABRB3* genes [5].

Transgenic Mice

As mentioned above, transgenic mice are created by different means to study the outcomes of misexpression or overexpression of gene products or in rescue studies. They are also used in crosses to generate tissue-specific gene targeting. In many instances, loss or gain-of-function mutations in a given gene generate malformations in the same structures [5], indicating a stringent

control of the amount and timing of gene activity. This is exemplified by orofacial clefting and craniofacial anomalies in mutant mice harboring tissue-specific loss-of- or gain-of-function alleles of *Smoothened*, encoding a non-redundant signal transducer of the Hedgehog signaling pathway [64, 73]. Similarly, loss of *Sonic Hedgehog* (*Shh*) in the palatal epithelium generates CP [63], whereas overexpression of *Shh* in the epithelia of transgenic embryos engenders a range of malformations, including orofacial clefting, tooth anomalies and skin tumors similar to those found in humans suffering from the nevoid basal cell carcinoma syndrome [74]. In *Shh*-overexpressing mice, increased amounts of Shh protein prevented palatal fusion by suppressing normal apoptosis in the MES [74].

The use of transgene expression to rescue lethal null mutations is another way to study the function of genes during lip and palate development. In this regard, homozygous mice lacking *Runx1*, encoding a transcription factor that is crucial for hematopoiesis and skeletogenesis, die at E12.5, prior to palatogenesis. *Runx1* is expressed in the palatal MEE [75]. Furthermore, studies suggest that humans with leukemias often display CL/CP, and that CL/CP patients are prone to develop leukemias [75 and references therein]. These observations led to implementation of a tissue-specific transgene rescue strategy to explore the role of *Runx1* during palate development [75]. *Runx1* null mutant mice, which also harbor a *Runx1* transgene expressed under the control of the *GATA-1* promoter that targets the transgene to hematopoietic cells (*Runx1*^{-/-}; *Gata1-Runx1*), were found to escape the early embryonic lethality. However, they developed a localized anterior palatal clefting caused by failed fusion between the primary and secondary palates and between the palate and epithelium of the nasal septum [75]. Transgene rescue experiments can also provide information on the importance of strict control of dosage of proteins in a given developmental process. Mice with a large deletion

of chromosome 14 that includes the *Spry2* gene (*Pub36*^{-/-}) exhibit CP due to increased cell proliferation and disrupted expression patterns of factors regulated by FGF signaling [76]. *Spry2* encodes a member of the Sprouty family, which controls receptor tyrosine kinase signaling, including inhibition of ERK activation by FGF receptor signaling. However, mutants with targeted disruption of *Spry2* have no clefting phenotype [76]. This conundrum was solved by the findings that *Pub36*^{-/-} mutants that also carry a *BAC Spry2* transgene were rescued from developing palatal clefting as a result of low levels of *Spry2* expression. Thus, normal palatogenesis requires optimal amounts of *Spry2* gene product [76].

Mouse Models Generated by Insertional and ENU-Induced Mutagenesis

Gene trappings consist essentially of random insertions creating disruption of a genetic locus by a reporter gene, which at the same time provides a readout for the expression patterns of the disrupted gene [53]. A gene trap insertion disrupting the function of *Sumo1* has been informative as the *Sumo1*^{+/^{gt}} mutants, to a large extent, recapitulated the clefting phenotype in a patient with *SUMO1* haploinsufficiency [77]. SUMO1 belongs to a family of small ubiquitin-related modifiers which, following covalent attachment, induce protein sumoylation. The critical role for SUMO1 in lip and palate development is not only reflected by the devastating clefting following loss-of-function of one allele, but also by findings that it sumoylates a range of direct and indirect critical players during lip and palate development, including, SMAD4, MSX1, SOX9, EYA1, SATB2, TBX22, p53 and p63 proteins [5]. Mice heterozygous and homozygous for a gene trap null allele of *Irf6* [78], together with knock-in mutants carrying a common mutation in PPS [10, 39, 79], demonstrated a crucial function for IRF6 in the embryonic ectoderm and its derivatives.

A novel player during palatogenesis has been uncovered by a gene trap-induced insertion

disrupting the function of the gene encoding basonuclein 2 (*bnc2*), a zinc finger protein shown to be orthologous to the disco proteins of *Drosophila* [80]. Its paralog basonuclein 1 is involved in proliferation of keratinocytes of stratified squamous epithelia [81] and is expressed by gonadal germ cells [82]. *Bnc2* homozygous mutants have abnormally small heads and display CP due to deficient growth of the elevated PS, as well as craniofacial bone anomalies. In the developing palate, β -galactosidase staining portraying *bnc2* distribution revealed that the highest activity was confined to the mesenchyme of the PS of the anterior segment of the palate prior to E14.5, which after E16.5 extended to the entire palate [80]. This, together with evidence showing reduced cell proliferation, especially in the β -galactosidase-positive domains of the palatal mesenchyme of *bnc2* mutants [80], indicates a requirement for basonuclein 2 for proper outgrowth of the PS.

Random insertions can also be produced with DNA transposons, such as the mariner *Sleeping Beauty* (SB) reconstructed from fish or the insect *piggy Bac* (PB), which function in mammals using the principle of gene traps. SB and PB are DNA transposons that use the 'cut-and-paste' strategy to move about the genome. Transposons can be used as vectors in somatic and germline transgenesis or in phenotype-driven, forward genetics approaches. DNA transposons consist of a binary system using at the beginning two transgenic mice, the female expresses a transposase such as SB in the germline, while the male harbors transposons containing gene trap cassettes equipped with reporter genes. Thereafter, the F1 double transgenic males are repeatedly crossed with wild-type females. This leads to segregation of the different insertional events that occurred in their sperm in separate F2 animals. The SB transposon system has further been improved by the advent of the hyperactive SB transposase (SB100X) [52].

Human genetics studies combined with mouse models are powerful means to dissect the

genetic basis of congenital malformations [5]. This approach has been implemented in two recent studies which identified homozygous mutations in *SMOC1*, encoding Sparc-related modular calcium-binding protein 1 (SMOC1), as a cause of the rare autosomal recessive Waardenburg anophthalmia syndrome (WAS) [83, 84], a finding also reported in another study [85]. WAS is also known as ophthalamo-acromelic syndrome affecting development of the eyes (anophthalmia, microphthalmia) and limbs (post-axial oligodactyly, syndactyly). Mutant mice with *Smoc1* loss-of-function created by gene targeting [84] or by the use of the SB transposon-induced gene trap system [83] to a large extent recapitulated the WAS anomalies. Interestingly, a proportion of *Smoc1* homozygous mutants in both studies were reported to exhibit CP [83, 84], and CP seems to be a common feature in humans with WAS [84]. In *Smoc1* mutants, although the PS is elevated, they were unable to meet at the midline. The expression patterns of *Smoc1* in the developing limbs indicate a function at different stages of limb development, including patterning, precartilaginous condensation and apoptotic removal of interdigital tissue [83]. The latter has been found to be diminished together with altered expression of *Bmp2*, *Bmp7* and *Msx2* [83]. Although the function of SMOC1 is at present unclear, this glycoprotein belongs to the SPARC matricellular protein family believed to mediate cell-extracellular matrix interactions, and studies in *Xenopus* indicate that SMOC1 functions as a BMP antagonist [86].

The discovery of RNA interference (RNAi) elicited the emergence of a new era in biomedical research. At present, sophisticated methods enable the use of this strategy to knock-down gene expression not only in cell cultures, but also in model organisms, including the mouse [87]. Both lentiviral infection and the transposon system have been implemented to create RNAi knock-down of gene expression in transgenic mice [88, 89]. A recent study used these strategies to induce

transient transgenic (manipulated embryos are studied directly and are not used for establishment of transgenic lines) targeted RNAi knock-down of *Prdm 16* expression in mouse embryos to test their efficiencies as rapid approaches for studies of gene function [90]. The *Prdm 16* gene has been shown by the same group as the gene disrupted in *cleft secondary palate 1 (csp1)* mutants generated by ENU mutagenesis. ENU-induced mutagenesis is a powerful means that uses the random chemical mutagen ENU to generate, on a large scale, mice harboring point mutations that can lead to loss-of-function, gain-of-function, or dominant negative effects. Thus, ENU-derived alleles are valuable as they allow comparison with null alleles and unveil crucial amino acids for normal protein function. ENU can also generate very small deletions (20–50 bp) [13, 14]. *csp1* homozygous mutants as well as *Prdm 16* null mutants generated by gene trap disruption of the gene (*Prdm 16^{Gt683Lex}*) display essentially similar phenotypes, including CP due to failure of elevation of the PS as well as other craniofacial and non-craniofacial anomalies [91]. *Prdm 16* encodes a zinc finger transcriptional cofactor which, at least as indicated by in vitro assays, seems to modulate TGF- β signaling [91].

Embryos generated following lentiviral infections or the use of the non-viral *SB* or *PB* transposon systems for expression of *Prdm 16*-specific short hairpin RNAs (shRNAs) leading to reduced or loss of *Prdm 16* expression indicated that the *PB* transposon system was the best. Indeed the *PB* system allowed not only high transgenic efficiency, but also the generation of a high number of embryos that recapitulated the defects in *csp1* and *Prdm 16^{Gt683Lex}* homozygotes as compared to the *SB* system and lentiviral infection [90]. The latter two enabled low transgenesis/high phenotypic penetrance and high transgenesis/low phenotypic penetrance, respectively. These findings indicate that transient transgenic RNAi knock-down of gene expression is an efficient means to determine gene function in vivo

with the advantage of being a rapid strategy as compared to the use of homologous recombination in ES cells.

Studies of *csp1* and *Prdm 16^{Gt683Lex}* homozygous embryos revealed reduced TGF- β and BMP signalings in the structures expressing the gene trap reporter, including the PS, tongue and Meckel's cartilage [91]. Thus, given the known involvement of the TGF- β and BMP signalings during craniofacial development, altered TGF- β and/or BMP pathway activities are likely to contribute to the malformations in the *csp1* and *Prdm 16^{Gt683Lex}* mutants. Another recently identified mouse mutant, *cleft palate 1 (clft1)* created by ENU mutagenesis, has been shown to harbor a missense mutation in the coding region of *Irf6* leading to an amino acid change which replicates that found in a VWS family [92], making it a suitable model for VWS. Compared to the previously generated null and PPS *Irf6* alleles [78, 79], *Irf6^{clft1}* seems to be a hypomorphic allele [92].

Mouse mutations that replicate those found in humans are indeed invaluable for studying the functional and phenotypic outcomes of genetic alterations. Abnormal activation of a developmental factor is not necessarily the opposite of its loss-of-function, since similar outcomes such as CL/CP can result from altered cellular functions [5]. Furthermore, as has been recently shown, an activating mutation of *FGFr2* (*Fgfr2^{C342Y}*) in a mouse model of Crouzon syndrome does not necessarily translate into a gain-of-function phenotype, and in this particular case, negative regulatory mechanisms may likely be involved [93]. FGF signaling operates at different levels during lip and palate development, and both activating and loss-of-function mutations generate CP [5]. The *Fgfr2^{C342Y}* allele, which led to high and low incidence of CP in homozygous and heterozygous mutants, respectively, due to altered outgrowth and delayed PS elevation, is a good model to dissect not only the role of FGFR2 signaling during palatogenesis but also the mechanisms of PS elevation [93].

Avoiding the Mouse Trap

To hype the mouse as the best model organism for CL and CP research is correct, however a few caveats and disadvantages should be taken into consideration when using the mouse to study biological phenomena during normal and abnormal lip and palate development [5].

Scarcity of Mouse Models for CL/CP and Differences between Humans and Mice

Notwithstanding surmountable issues such as early embryonic lethality and severe malformations which hinder studies of gene function during lip and palate development, the most remarkable frustration with mice is the dearth of mutants with CL/CP as compared to those with CPO. In the former, the clefting is usually accompanied by major craniofacial defects owing to anomalies of cranial neural crest cells and their descendants or to disruption of factors, such as SUMO1, which control the expression and activity of a multitude of genes and proteins that are crucial for lip and primary palate development. This situation has also been encountered in teratogen-induced orofacial clefts in mice where, in contrast to induction of CPO, only mouse strains known to be susceptible of developing spontaneous CL/CP display CL/CP following treatment [5]. Plausible causes for the scarcity of mutants with CL/P as compared to CPO may reside in the fact that lip and primary palate ontogenesis is separate from that of the secondary palate, and/or may involve genes and networks that are to a large extent different from those operating during palate development [5, 6]. Further compounding the problem, and with rare exceptions, is the occurrence of CPO in mice following inactivating mutations, whereas in humans the mutated genes, whether in syndromic or isolated clefting conditions lead to CL/CP. Here the problem may reside in the fact that the functional outcomes (reduced activity, loss-of-function, or altered function) of mutated genes in humans are to a large extent unknown

[5, 94]. Actually, we are sometimes hit with the unexpected, as illustrated in a known activating mutation of *Fgfr2* which turned out to translate into loss of FGF signaling in the developing mouse palate [93]. Another possible cause of the rarity of CL/CP vs. CPO mouse models could be the relatively short time of development in mice of the upper lip and primary palate (≈ 2.5 days) as compared to the secondary palate ($\approx 4\text{--}4.5$ days), with the latter also being under the influence of other head structures. However, in the human embryo, development of the upper lip and primary palate (from the 4th to the 7th week of gestation) is relatively long, thus functional alterations of orthologs have the time to translate into CL/CP.

Several mutant mice for genes known to be causal for clefting in humans are either exempt from clefting, display a more severe phenotype or only a fraction of the phenotype. Notwithstanding a handful of mutants [5], the majority of genes disrupted in mice generate a clefting phenotype only upon alteration of the two copies, whereas in humans haploinsufficiency leads to clefts [5]. In addition, several conditions in humans, such as those derived from loss-of-function mutations of *FLNA*, encoding filamin A, as well as haploinsufficiencies in one or multiple contiguous genes at 17p13.3 such as *HIC1*, *OVCA1*, *CRK* and *MNT* known or proposed to be amongst those involved in Miller-Dieker syndrome, lead to a range of malformations. However, the affected patients are exempt of cleftings, whereas homozygous null mutations of their orthologs in mice generate CPO with other anomalies [5]. Influences such as genetic background and environmental factors, functional redundancy or involvement of compensatory loci, modifier genes or the nature of the mutations can lead to these differences. Finally, when making analogies with non-syndromic clefting conditions in humans, it is worth keeping in mind that the majority if not all mouse models for CL/CP and CPO display other non-clefting major or minor anomalies.

Others Caveats

Differences between mouse models for orofacial clefting have been observed in mutants generated by similar strategies to disable a given gene due to differences in the genetic background of the strains used. These may result from influences by modifier loci. Others, despite being on the same genetic background, display different phenotypes as a result of differences in the targeting strategies or allele differences. Reporter mice such as those used to localize the sites of active canonical Wnt signaling can be misleading, since in some instances reporter activity is absent (probably due to difficulties in detection of weak activity) from true sites of Wnt- β -catenin signaling, whereas in others reporter activity is present at locations devoid of this signaling pathway. Similarly, experiments using *lacZ* reporter mice for cell fate mappings can lead to misinterpretations in the case of erroneous staining of tissues. This can happen for example upon the use of low pH of the staining buffer (reaction with endogenous β -galactosidase in tissues such as epithelia and the mesenchyme) or inadequate processing of specimens before, during and after staining.

Concluding Remarks

Mouse models, whether engineered to specifically study the function, regulation and targets of factors known to be involved in lip and palate development and clefting, or mutants in which the discovery of a gene operating during development

of these structures is fortuitous, have revealed an overwhelming number of key players. So many genes are involved in growth, morphogenesis and/or fusion of lip and palate primordia, yet disruption of one gene is disastrous. Mouse models taught us that the complexity of building relatively simple anatomical structures such as the lip and palate is not limited to the sheer number of factors involved, but is mainly due to how distinct proteins or proteins belonging to the same family cooperate and/or display antagonizing activities as development proceeds. Thus, stringent and complex spatiotemporal molecular control of cellular processes is key to normal development. Given the pace of technological prowesses, the dawn of creation of thousands of novel mutants, the advances in human genetics research, as well as the ease with which nowadays scientists can gather information, the near future may provide a clearer understanding of the molecular web that governs the behavior and fate of the cells which make up the lip and palate. Thus, despite a few surmountable disadvantages, the mouse remains the model of choice in CL and CP research.

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References

- 1 Skarnes WC, Rosen B, West AP, Koutsourakis M, Bushell W, Iyer V, Mujica AO, Thomas M, Harrow J, Cox T, Jackson D, Severin J, Biggs P, Fu J, Nefedov M, de Jong PJ, Stewart AE, Bradley A: A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* 2011;474:337342.
- 2 Dudas M, Kaartinen V: Tgf-beta superfamily and mouse craniofacial development: interplay of morphogenetic proteins and receptor signaling controls normal formation of the face. *Curr Top Dev Biol* 2005;66:65–133.
- 3 Jiang R, Bush JO, Lidral A: Development of the upper lip: morphogenetic and molecular mechanisms. *Dev Dyn* 2006;1152–1166.
- 4 Gritli-Linde A: Molecular control of secondary palate development. *Dev Biol* 2007;301:309–326.

- 5 Gritli-Linde A: The etiopathogenesis of cleft lip and cleft palate: usefulness and caveats of mouse models. *Curr Top Dev Biol* 2008;84:37–138.
- 6 Juriloff DM, Harris MJ: Mouse genetic models of cleft lip with or without cleft palate. *Birth Defects Res A Clin Mol Teratol* 2008;82:63–77.
- 7 Chai Y, Maxton RE: Recent advances in craniofacial morphogenesis. *Dev Dyn* 2006;235:2353–2375.
- 8 Huang X, Goudy SL, Ketova T, Litingtung Y, Chiang C: *Gli3*-deficient mice exhibit cleft palate associated with abnormal tongue development. *Dev Dyn* 2008;237:3079–3087.
- 9 Liu W, Lan Y, Pauws E, Meester-Smoor M, Stanier P, Zwarthoff EC, Jiang R: The *Mn1* transcription factor acts upstream of *Tbx22* and preferentially regulates posterior palate growth in mice. *Development* 2008;135:3959–3968.
- 10 Thomason HA, Zhou H, Kouwenhoven EN, Dotto G-P, Restivo G, Nguyen B-C, Little H, Dixon MJ, van Bokhoven H, Dixon J: Cooperation between the transcription factors p63 and IRF6 is essential to prevent cleft palate in mice. *J Clin Invest* 2010;120:1561–1569.
- 11 Xu X, Han J, Ito Y, Bringas P Jr, Deng C, Chai Y: Ectodermal and p38MAPK are functionally redundant in mediating TGF- β /BMP signaling during tooth and palate development. *Dev Cell* 2008;15:322–329.
- 12 He F, Xiong W, Yu X, Espinoza-Lewis R, Liu C, Gu S, Nishita M, Suzuki K, Yamada G, Minami Y, Chen UP: Wnt5a regulates directional cell migration and cell proliferation via Ror2-mediated noncanonical pathway in mammalian palate development. *Dev Biol* 2008;135:3871–3879.
- 13 Papaioannou VE, Behringer RR: Mouse phenotypes; in Papaioannou VE, Behringer RR (eds): *A Handbook of Mutation Analysis*. New York, Cold Spring Harbor Laboratory Press, 2005.
- 14 Nagy A, Gertsenstein M, Vintersten K, Behringer R: Manipulating the mouse embryo; in Nagy A, Gertsenstein M, Vintersten K, Behringer R (eds): *A Laboratory Manual*, ed 3, New York, Cold Spring Harbor Laboratory Press, 2003.
- 15 Pauws E, Hoshino A, Bentley L, Prajapati S, Keller C, Hammond P, Martinez-Barbera J-P, Moore GE, Stanier P: *Tbx22* null mice have a submucous cleft palate due to reduced palatal bone formation and also display ankyloglossia and choanal atresia. *Hum Mol Genet* 2009;18:4171–4179.
- 16 Pasquale EB: Eph-ephrin bidirectional signaling in physiology and disease. *Cell* 2008;133:38–52.
- 17 Davy A, Aubin J, Soriano P: Ephrin-B1 forward and reverse signaling are required during mouse development. *Genes Dev* 2004;18:572–583.
- 18 Orioli D, Hekemeyer M, Lemke G, Klein R, Pawson T: *Sek4* and Nuk receptors cooperate in guidance of commissural axons and in palate formation. *EMBO J* 1996;15:6035–6049.
- 19 Bush JO, Soriano P: Ephrin-B1 forward signaling regulates craniofacial morphogenesis by controlling cell proliferation across Eph-ephrin boundaries. *Genes Dev* 2010;24:2068–2080.
- 20 Risley M, Garrod D, Henkemeyer M, McLean W: EphB2 and EphB3 forward signalling are required for palate development. *Dev Biol* 2009;126:230–239.
- 21 Bush JO, Soriano P: Ephrin-B1 regulates axon guidance by reverse signaling through a PDZ-dependent mechanism. *Genes Dev* 2009;23:1586–1599.
- 22 Compagni A, Logan M, Klein R, Adams RH: Control of skeletal patterning by EphrinB1-EphB interactions. *Dev Cell* 2003;5:217–230.
- 23 Cho K-W, Cho S-W, Lee J-M, Lee M-J, Gang H-S, Jung H-S: Expression of phosphorylated forms of ERK, MEK, PTEN and PI3K in mouse oral development. *Gene Exp Patterns* 2008;8:284–290.
- 24 Newbern J, Zhong J, Wickramasinghe R, Li X, Wu Y, Samuels I, Cherosky N, Karlo IC, O'Loughlin B, Wikenheiser J, Gargasha M, Doughman YQ, Charron J, Ginty DD, Watanabe M, Saitta SC, Snider WD, Landreth GE: Mouse and human phenotypes indicate critical conserved role for ERK signaling in neural crest development. *Proc Natl Acad Sci USA* 2008;105:17115–17120.
- 25 Goudy S, Law A, Sanchez G, Baldwin HS, Brown C: *Tbx1* is necessary for palatal elongation and elevation. *Mech Dev* 2010;127:292–300.
- 26 Thomason HA, Dixon MJ, Dixon J: Facial clefting in *Tp63*-deficient mice result from altered Bmp4, Fgf8 and Shh signaling. *Dev Biol* 2008;321:273–282.
- 27 Nakatomi M, Wang XP, Key D, Lund JJ, Turbe-Doan A, Kist R, Aw A, Chen Y, Maas RL, Peters H: Genetic interactions between *Pax9* and *Msx1* regulate lip development and several stages of tooth morphogenesis. *Dev Biol* 2010;340:438–449.
- 28 Gao Y, Lan Y, Ovitt CE, Jiang R: Functional equivalence of the zinc finger transcription factors *Osr1* and *Osr2* in mouse development. *Dev Biol* 2009;328:200–209.
- 29 Lan Y, Ovitt CE, Cho ES, Maltby KM, Wang Q, Jiang R: *Odd-skipped related 2* (*Osr2*) encodes a key intrinsic regulator of secondary palate growth and morphogenesis. *Development* 2004;131:3207–3216.
- 30 Jin J-Z, Li Q, Higashi Y, Darling DS, Ding J: Analysis of *Zfhx1a* mutant mice reveals shelf contact independent medial edge epithelial differentiation during palate fusion. *Cell Tissue Res* 2008;333:29–38.
- 31 Kinoshita M, Era T, Jakt LM, Nishikawa S-I: The novel protein kinase Vlk is essential for stromal function of mesenchymal cells. *Development* 2009;136:2069–2079.
- 32 Enomoto H, Nelson CM, Somerville RPT, Mielke K, Dixon LJ, Powell K, Apte SS: Cooperation of two ADAMTS metalloproteinases in closure of the mouse palate identifies a requirement for versican proteolysis in regulating palatal mesenchyme proliferation. *Development* 2010;137:4029–4039.
- 33 Hilliard SA, Yu L, Gu S, Zhang Z, Chen YP: Regional regulation of palatal growth and patterning along the anterior-posterior axis in mice. *J Anat* 2005;207:655–667.
- 34 Glossl J, Hoppe W, Kresse H: Post-translational phosphorylation of proteodermatan sulfate. *J Biol Chem* 1986;350:1920–1923.
- 35 Apte SS: A disintegrin-like and metalloproteinase (reprolysin-type) with thrombospondin type 1 motif (ADAMTS) superfamily: functions and mechanisms. *J Biol Chem* 2009;284:31993–31997.

- 36 Vaziri Sani F, Kaartinen V, El Shahawy M, Linde A, Gritli-Linde A: Developmental changes in cellular and extracellular structural macromolecules in the secondary palate and in the nasal cavity of the mouse. *Eur J Oral Sci* 2010;118:221–236.
- 37 Mjaatvedt, CH, Yamamura H, Capehart AA, Turner D, Markwald RR: The *Cspg2* gene disrupted in the *hdf* mutant, is required for right cardiac chamber and endocardial cushion formation. *Dev Biol* 1998;202:56–66.
- 38 Venza I, Visalli M, Parrillo L, De Felici M, Teti D, Venza M: *MSX1* and *TGF- β 3* are novel target genes functionally regulated by *FOXE1*. *Hum Mol Genet* 2011; 20:1016–1025.
- 39 Moretti F, Marinari B, Lo Lacono N, Botti E, Giunta A, Spallone G, Garaffo G, Vernersson-Lindahl E, Merlo G, Mills AA, Ballaró C, Alemà S, Chimenti S, Guerrini L, Constanzo A: A regulatory feedback loop involving *p63* and *IRF6* links the pathogenesis of two genetically different human ectodermal dysplasias. *J Clin Invest* 2010;120:1570–1577.
- 40 Gritli-Linde A: *p63* and *IRF6*: brothers in arms against cleft palate. *J Clin Invest* 2010;120:1386–1388.
- 41 Richardson RJ, Dixon J, Jiang R, Dixon MJ: Integration of *IRF6* and *Jagged2* signalling is essential for controlling palatal adhesion and fusion competence. *Hum Mol Genet* 2009;18:2632–2642.
- 42 Murray SA, Oram KF, Gridley T: Multiple functions for snail family genes during palate development in mice. *Development* 2007;134:1789–1797.
- 43 Aluwihare P, Mu Z, Zhao Z, Yu D, Weinreb PH, Horan GS, Violette SM, Munger JS: Mice that lack activity of $\alpha v\beta 6$ - and $\alpha v\beta 8$ -integrins reproduce the abnormalities of *Tgfb1*- and *Tgfb3*-null mice. *J Cell Sci* 2009;122:227–232.
- 44 Choi KY, Kim HJ, Cho BC, Kim IS, Kim HJ: A *TGF- β* -induced gene, β ig-h3 is crucial for apoptotic disappearance of the medial edge epithelium in palate fusion. *J Cell Biochem* 2009;107:818–825.
- 45 Kim JE, Kim SJ, Jeong HW, Lee BH, Choi JY, Park RW, Park JY, Kim IS: RGD peptides released from β ig-h3, a *TGF- β* -induced cell adhesive molecule, mediate apoptosis. *Oncogene* 2003;22:2045–2053.
- 46 Davis H, Lewis A, Spencer-Dene B, Tateossian H, Stamp G, Behrens A, Tomlinson I: *FBXW7* mutations typically found in human cancers are distinct from null alleles and disrupt lung development. *J Pathol* 2011;224:180–189.
- 47 Tateossian H, Hardisty RE, Morse S, Romero MR, Hilton H, Dean C, Brown SDM: Regulation of *TGF- β* signaling by *Fbxo11*, the gene mutated in the Jeff otitis media mouse mutant. *Pathogenetics* 2009;2:5.
- 48 Uetani N, Bertozzi K, Chagnon M, Hendriks W, Tremblay ML, Bouchard M: Maturation of ureter-bladder connection in mice is controlled by *LAR* family receptor protein tyrosine phosphatases. *J Clin Invest* 2009;119:924–935.
- 49 Hahn P, Lindsten T, Tolentino M, Thompson CB, Bennett J, Dunaief JL: Persistent fetal ocular vasculature in mice deficient of *Bax* and *Bak*. *Arch Ophthalmol* 2005;123:797–802.
- 50 Xu X, Han J, Yoshihiro I, Bringas P Jr, Urata MM, Chai Y: Cell autonomous requirement for *Tgfb2* in the disappearance of medial edge epithelium during palatal fusion. *Dev Biol* 2006;297:238–248.
- 51 Mooney RA, LeVea CM: The leucocyte common antigen-related protein *LAR*: candidate PTP for inhibitory targeting. *Curr Top Med Chem* 2003;3:809–819.
- 52 Ivics Z, Li MA, Mátés L, Boeke JD, Nagy A, Bradley A, Izsvák Z: Transposon-mediated genome manipulation in vertebrates. *Nat Methods* 2009;6:415–422.
- 53 Stanford WL, Cohn JB, Cordes SP: Gene-trap mutagenesis: past, present and beyond. *Nat Rev Genet* 2001;2:756–768.
- 54 Rossant J, McMahon A: 'Cre'-ating mouse mutants: a meeting review on conditional mouse genetics. *Genes Dev* 1999;13:142–145.
- 55 Song L, Li Y, Wang K, Wang YZ, Molotov A, Gao L, Zhao T, Yamagami T, Wang Y, Gan Q, Pleasure DE, Zhou CJ: *Lrp6*-mediated canonical *Wnt* signaling is required for lip formation and fusion. *Development* 2009;136:3161–3171.
- 56 Yang X, Kilgallen S, Andreeva V, Spicer DB, Pinz I, Friesel R: Conditional expression of *Spry1* in neural crest causes craniofacial and cardiac defects. *BMC Dev Biol* 2010;10:48.
- 57 Wu M, Li J, Engleka KA, Zhou B, Lu MM, Plotkin JB, Epstein JA: Persistent expression of *Pax3* in neural crest causes cleft palate and defective osteogenesis in mice. *J Clin Invest* 2008;118:2076–2087.
- 58 Li WY, Dudas M, Kaartinen V: Signaling through *Tgf- β* type I receptor *Alk5* is required for upper lip fusion. *Mech Dev* 2008;125:874–882.
- 59 Li L, Lin M, Wang Y, Cserjesi P, Chen Z, Chen YP: *Bmpr1a* is required in mesenchymal tissue and has limited redundant function with *Bmpr1b* in tooth and palate development. *Dev Biol* 349:451–461.
- 60 Baek JA, Lan Y, Liu H, Maltby KM, Mishina Y, Jiang R: *Bmpr1a* signaling plays critical roles in palatal shelf growth and palatal bone formation. *Dev Biol* 2011;350:520–531.
- 61 He F, Xiong W, Wang Y, Li L, Liu C, Yamagami T, Makoto MT, Zhou C, Chen YP: Epithelial *Wnt/ β -catenin* signaling regulates palatal shelf fusion through regulation of *Tgfb3* expression. *Dev Biol* 2011;350:511–519.
- 62 Hosokawa R, Deng X, Takamori K, Xu X, Urata M, Bringas P Jr, Chai Y: Epithelial-specific requirement of *FGFR2* signaling during tooth and palate development. *J Exp Zool B Mol Dev Evol* 2009;312B: 343–350.
- 63 Rice R, Spencer-Dene B, Connor EC, Gritli-Linde A, McMahon AP, Dickson C, Theseleff I, Rice DP: Disruption of *Fgf10/Fgfr2b*-coordinated epithelial-mesenchymal interactions causes cleft palate. *J Clin Invest* 2004;113:1692–1700.
- 64 Lan Y, Jiang R: Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth. *Development* 2009;136:1387–1396.
- 65 He F, Popkie AP, Xiong W, Li L, Wang Y, Phiel CJ, Chen Y: *Gsk3 β* is required in the epithelium for palatal elevation in mice. *Dev Dyn* 2010;239:3235–3246.
- 66 Xiong W, He F, Morikawa Y, Yu X, Zhang Z, Lan Y, Jiang R, Cserjesi P, Chen Y: *Hand2* is required in the epithelium for palatogenesis in mice. *Dev Biol* 2009;330:131–141.

- 67 Zouvelou V, Luder HU, Mitsiadis TA, Graf D: Deletion of BMP7 affects the development of bones, teeth and other ectodermal appendages of the orofacial complex. *J Exp Zool B Mol Dev Evol* 2009;312B:361–374.
- 68 Wyatt AW, Osborne RJ, Stewart H, Ragge NK: Bone morphogenetic protein 7 (*BMP7*) mutations are associated with variable ocular, brain, ear, palate and skeletal anomalies. *Hum Mutat* 2010; 31:781–787.
- 69 Bakrania P, Efthymiou M, Klein JC, Salt A, Bunyan DJ, Wyatt A, Ponting CP, Martin A, Williams S, Lindley V, Gilmore J, Restori M, Robson AG, Neveu MM, Holder GE, Collin RO, Robinson DO, Fardorn P, Johansen-Berg H, Gerrelli D, Ragge NK: Mutations in *BMP4* cause eye, brain, and digit developmental anomalies: overlap between *BMP4* and Hedgehog signaling pathways. *Am J Hum Genet* 2008;82:304–319.
- 70 Suzuki S, Marazita ML, Cooper ME, Miwa N, Hing A, Jugessur A, Natsume N, Shimozato K, Ohbayashi N, Suzuki Y, Niimi T, Minami K, Yamamoto M, Altannamar TJ, Erkhembataar T, Furukawa H, Daack-Hirsch S, L'heureux J, Brandon CA, Weinberg SM, Neiswanger K, Deleyiannis FW, de Salamanca JE, Vieira AR, Lidral AC, Martin JE, Murray JC: Mutations in *BMP4* are associated with subepithelial, microform, and over cleft lip. *Am J Hum Genet* 2009;84:406–411.
- 71 Liu W, Sun X, Braut A, Mishina Y, Behringer RR, Mina M, Martin JE: Distinct functions of Bmp signaling in lip and palate fusion in mice. *Development* 2005;132:1453–1461.
- 72 Oh WJ, Westmoreland JJ, Summers R, Condie BG: Cleft palate is caused by CNS dysfunction in *Gad1* and *Viaa* knockout mice. *PLoS One* 2010;5: e9758.
- 73 Jeong J, Mao J, Tenzen T, Kottman AH, McMahon AP: Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial promordia. *Genes Dev* 2004;18:937–951.
- 74 Cobourne MT, Xavier GM, Depew M, Hagan L, Sealby J, Webster Z, Sharpe PT: Sonic hedgehog signalling inhibits palatogenesis and arrest tooth development in a mouse model of the nevoid basal cell carcinoma syndrome. *Dev Biol* 2009;331:38–49.
- 75 Charoenchaikorn K, Yokomizo T, Rice DP, Honjo T, Matsuzaki K, Shintaku Y, Imai Y, Wakamatsu A, Takahashi S, Ito Y, Takano-Yamamoto T, Thesleff I, Yamamoto M, Yamashiro T: *Runx1* is involved in the fusion of the primary and secondary palatal shelves. *Dev Biol* 2009; 326:392–402.
- 76 Welsh IC, Hagge-Greenberg A, O'Brien TP: A dosage-dependent role for *Spry2* in growth and patterning during palate development. *Mech Dev* 2007;124:746–761.
- 77 Alkuraya F, Saadi I, Lund J, Turbe-Doan A, Morton CC, Maas RL: *SUMO1* haploinsufficiency leads to cleft lip and palate. *Science* 2006;313:1751.
- 78 Ingraham CR, Kinoshita A, Kondo S, Yang B, Sajjan S, Trout KJ, Malik MI, Dunnwald M, Goudy SL, Lovett M, Murray JC: Abnormal skin, limb and craniofacial morphogenesis in mice deficient for interferon regulatory factor 6 (*Irf6*). *Nat Genet* 2006;38:1335–1340.
- 79 Richardson RJ, Dixon J, Malhotra S, Hardman MJ, Knowles L, Boot-Handford RP, Shore P, Witmark A, Dixon MJ: *Irf6* is a key determinant of the keratinocyte proliferation-differentiation switch. *Nat Genet* 2006;38:1329–1334.
- 80 Vanhottetghem A, Maciejewski-Duval A, Bouche C, Delhomme B, Hervé F, Daubigney F, Soubigou G, Araki M, Araki K, Yamamura KI, Dijian P: *Basonuclin 2* has a function in the multiplication of embryonic craniofacial mesenchymal cells and is orthologous to disco proteins. *Proc Natl Acad Sci USA* 2009;106: 14432–14437.
- 81 Tseng H, Green H: *Basonuclin*: A keratinocyte with multiple paired zinc fingers. *Proc Natl Acad Sci USA* 1992;89:10311–10315.
- 82 Yang Z, Gallicano G, Yu Q, Fuchs E: An unexpected localization of *basonuclin* in the centrosome, mitochondria, and acrosome of developing spermatids. *J Cell Biol* 1997;137:657–669.
- 83 Okada I, Hamanoue H, Terada K, Tohma T, Megarbane A, Chouery E, Abou-Gosh J, Jalkh N, Cogulu O, Ozkinay F, Horie K, Takeda J, Furuichi T, Ikegawa S, Nishiyama K, Miyatake S, Nishimura A, Mizuguchi T, Niikawa N, Hirahara F, Kaname T, Yoshiura KI, Tsurusaki Y, Doi H, Miyake N, Furukawa T, Matsumoto N, Saitsu H: *SMOC1* is essential for ocular and limb development in humans and mice. *Am J Hum Genet* 2011;88:30–41.
- 84 Rainger J, van Beusekom E, Ramsey JK, McKie L, Al Ghazali L, Pallotta R, Saponari A, Branney P, Fisher M, Morrison H, Binknell L, Gautier P, Perry P, Sokhi K, Sexton D, Bardakjian TM, Schneider AS, Elcioglu N, Ozkinay F, Koenig R, Megarbane A, Semerci CN, Khan A, Zafar S, Hennekam R, Sousa SB, Ramos L, Garavelli L, Superti Furga A, Wichmeijer A, Jackson JJ, Gillesen-Kaesbach G, Brunner HG, Wieczorek D, van Bokhoven H, FitzPatrick DR: Loss of the *BMP* antagonist, *SMOC1*, causes ophtalmo-acromelic (Waardenburg anophthalmia) syndrome in humans and mice. *PLoS Genet* 2011;7:e1002114.
- 85 Abouzeid H, Boisset G, Favez T, Youssef M, Marzouk I, Shakankiry N, Bayoumi N, Descombes P, Agosti C, Munier FL, Schorderet DF: Mutations in the *SPARC*-related modular calcium-binding protein1 gene, *SMOC1*, cause Waardenburg anophthalmia syndrome. *Am J Hum Genet* 2011;82:304–319.
- 86 Thomas JT, Canelos P, Luyten FP, Moos M Jr: *Xenopus* *SMOC-1* inhibits *BMP* signaling downstream of receptor binding and is essential for post-gastrulation development in *Xenopus*. *J Biol Chem* 2009;284:18994–19005.
- 87 Campeau E, Gobeil S: RNA interference in mammals: behind the screen. *Brief Funct Genomics* 2011;10:215–226.
- 88 Miskey C, Izsvak Z, Kawakami K, Ivics Z: DNA transposon in vertebrate functional genomics. *Cell Mol Life Sci* 2005;62:629–641.
- 89 Park F: Lentiviral vectors: are they the future of animal transgenesis? *Physiol Genomics* 2007;31:159–173.
- 90 Bjork BC, Fujiwara Y, Davis SW, Qiu H, Saunders TL, Sandy P, Orkin S, Camper SA, Beier DR: A transient transgenic RNAi strategy for rapid characterization of gene function during embryonic development. *PLoS One* 2010;5:e14375.

- 91 Bjork BC, Turbe-Doan A, Prysak M, Herron BJ, Beier DR: *Prdm 16* is required for normal palatogenesis in mice. *Hum Mol Genet* 2010;19:774–789.
- 92 Stottman RW, Bjork BC, Beier DR: Identification of a van der Woude syndrome mutation in the *cleft palate 1* mutant mouse. *Genesis* 2010;48:303–308.
- 93 Snider-Warwick AK, Perlyn CA, Pan J, Yu K, Zhang L, Ornitz DM: Analysis of gain-of-function FGFR2 Crouzon mutation provides evidence of loss of function activity in the etiology of CP. *Proc Natl Acad Sci USA* 2010;107:2515–2520.
- 94 Vieira AR: Unraveling human cleft lip and palate research. *J Dent Res* 2008;87:119–125.

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Hedgehog Signalling in Development of the Secondary Palate

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Abstract

The Sonic hedgehog (*Shh*) gene encodes a secreted signalling molecule that plays an important role during numerous aspects of vertebrate development. In the developing palate, *Shh* is strongly expressed in the epithelium on the oral surface, in a series of stripes corresponding to the future *rugae palatini*. There is now good evidence that Shh is involved in a number of signalling interactions that take place between the epithelium and mesenchyme during normal palatogenesis. In particular, being able to induce *Fgf10* in mesenchyme of the anterior palate which, via Fgfr2, is able to induce *Shh* in the epithelium. These interactions are essential for normal growth and development of this region; in the absence of normal Shh signalling, mice develop a cleft of the secondary palate. Growth and patterning of the secondary palate are closely linked, with successive rugae forming within a mid-palatal growth zone. Shh also plays a key role during this early patterning process, along the anteroposterior axis of the secondary palate. Specifically, acting as an inhibitor within a reaction-diffusion mechanism that is responsible for establishing primary architecture of the rugae.

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The Hedgehog family of secreted signalling molecules are of fundamental importance for normal embryonic development in numerous organisms [1, 2]. In vertebrates, Sonic hedgehog (Shh) plays

a key role during development of many regions within the embryo, including the craniofacial complex [3]. Signalling is mediated in target cells by binding of ligand to the Patched (Ptch1) twelve-pass transmembrane domain receptor [4], an interaction facilitated by several negatively regulated membrane-associated proteins, including Cdo, Boc and Gas1 [5–8]. Paradoxically, in the absence of ligand, Ptch1 actually inhibits pathway activity through the modulation of Smoothened (Smo) [9, 10], a seven-pass transmembrane-domain protein absolutely required for intracellular transduction [11]. However, binding of Shh to Ptch1 derepresses Smo and allows pathway activation, although as a direct transcriptional target of signalling, *Ptch1* rapidly mediates sequestration and degradation of Shh to re-establish quiescence in responding cells [12, 13]. This regulated buffering by Ptch1 influences both the concentration and duration of signal activity in determining the cellular response [12, 14]. Within the cell, signalling is mediated through the modification of Gli protein transcriptional activity [15–19]. Primarily, this occurs by preventing degradation of Gli2 (and Gli1) transcriptional activators [20] and promoting suitable processing of the Gli3 transcriptional repressor [21, 22]. In addition, it has become clear that normal Shh function also requires

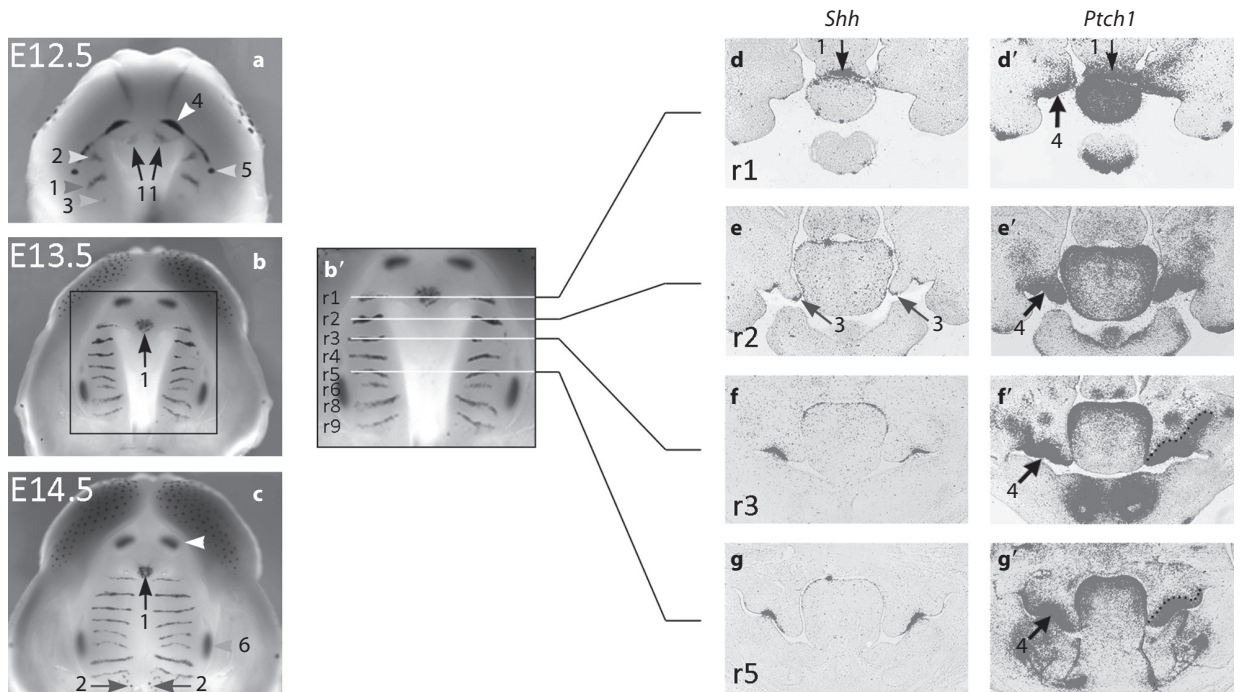


Fig. 1. *Shh* expression in the developing primary and secondary palate. **a–c** From E12.5 to 14.5, *Shh* is seen in epithelium of the midline primary palate (arrow 1), progressively in the developing rugae (r) of the secondary palate (highlighted in **b'**) beginning with (highlighted in **a**) r8 (arrowhead 1), then r2 (arrowhead 2) and r9 (arrowhead 3, just beginning to form); incisor tooth bud (arrowhead 4), vestigial (arrowhead 5) and first molar (arrowhead 6) tooth buds and sensory papilla of the soft palate (arrow 2). **d–g** At E13.5, prior to palatal shelf elevation, *Shh* is restricted to oral epithelium of the primary (arrow 1) and secondary palates, although expression in ruga 2 does extend to the medial edge epithelium (arrow 3). **d'–g'** At the same stage, *Ptch1* is expressed in the oral epithelium and mesenchyme of the primary palate (arrow 1 in **d'**) and mediolaterally along the oral region of the secondary palate shelves (arrow 4). Within the oral region, a gradient of transcriptional activity is present, progressively diminishing toward the nasal side, with loss of expression defining the oro-nasal boundary (dotted line in **f'** and **g'**).

the primary cilium [23, 24] and that fine control of Gli protein transcriptional activity takes place within this organelle; a process dependent upon normal intraflagellar (i.e. intracilium) transport [25–29].

***Shh* Expression in the Developing Palate**

Shh expression is seen initially in the palatal epithelium of the mouse embryo from around E12, in the midline of the primary palate and as a series of stripes that appear on the oral surface of

the secondary palate and correspond to the future *rugae palatini* [30, 31] (fig. 1). The appearance of these nine stripes occurs as the palatal shelves are growing, beginning with ruga 8 and closely followed by ruga 2. Rugae 3–7 form through reiterative and sequential interposition between the preceding ruga and ruga 8, with (the most anterior) ruga 1 appearing out of sequence, after ruga 3 [30]. This localized expression extends along the oral side of the medial edge epithelium in the anterior-most regions of the palate at E13.5 [32, 33], but no *Shh* expression is seen on the nasal side of the palate epithelium [34]. *Shh* is also expressed along

the posterior edge of the future soft palate from around E13.5, concentrating within the midline of this structure at E15 [35]. These expression domains are driven by two conserved non-coding sequences, MRCS1 and MFCS4, which are situated 600–900 kb upstream of the *Shh* transcriptional start site and control expression in the future hard and soft palate regions, respectively [35].

A number of components within the Shh pathway are also expressed in epithelium and mesenchyme of the palatal shelves throughout palatogenesis, indicating the likely presence of both interepithelial and epithelial-mesenchymal signalling during this process [36]. In particular, from E12.5 to 13.5, *Ptch1* and *Gli1* are both strongly expressed mediolaterally along the oral region of the palatal shelves. Within the oral region, a gradient of transcriptional activity is present, progressively diminishing toward the nasal side, with loss of expression defining the oronasal boundary [34] (fig. 1). During later stages of development between E14.5 and 15.5, the expression of *Ptch1* and *Gli1* localizes at the presumptive oral surface during elevation and fusion of the shelves; however, *Shh* expression is not seen in the medial epithelial seam during the process of fusion [34, 37, 38].

Targeted deletion of Shh has provided little direct information on the role of this signalling pathway during palatogenesis. This is largely due to a requirement for Shh during earlier craniofacial development. *Shh*^{-/-} mice have alobar holoprosencephaly and severe disruption of craniofacial skeletal elements [3]. The first pharyngeal arch in these mice is hypoplastic, fused in the midline and has severe restriction of growth in the maxillary and proximal mandibular arches [39]. Shh plays a key role in mediating growth and patterning of the early face, in particular through signalling to cranial neural crest cells maintaining their survival and proliferation. In the absence of signalling there is severe disruption of the facial skeleton with truncation and an absence of many cranial neural crest-derived skeletal and non-skeletal elements [40].

Role of Shh Signalling during Development of the Secondary Palate

The first evidence of a functional link between Shh and development of the secondary palate came from the analysis of *Msx1*^{-/-} mice. Anterior to the first molar tooth germ, Shh induces proliferation in isolated mesenchymal palatal shelf explants, indirectly through the induction of Bmp2 [41]. Moreover, *Msx1* activity is required in the mesenchyme for the expression of *Shh* in the overlying epithelium, through the induction of Bmp4 [41], signalling to the epithelium via upregulation of *Hand2* [32]. The finding that Shh can act as a mitogen in the palatal shelf mesenchyme has been corroborated [34, 42], with the maintenance of *Shh* expression in palatal epithelium also seemingly dependent upon Fgf10 in the mesenchyme, signalling through Fgfr2b in the epithelium [42].

The generation of mice with conditional inactivation of either *Shh* (*K14-Shh*^{c/n}) [43] or *Smo* (*K14-Smo*^{c/n}) [44] in the palatal epithelium has provided more direct evidence of a localized role for this pathway during growth of the secondary palate. *K14-Smo*^{c/n} mice, which lack interepithelial signalling, do not have a cleft palate [44], but abrogation of signalling from epithelium to mesenchyme in *K14-Shh*^{c/n} mice produces a significant cleft, associated with widely spaced and only rudimentary palatal shelves [38, 42]. However, penetrance of the *K14-Shh*^{c/n} mouse cleft varies between 70 and 85%, depending upon the *K14-Cre* transgenic mouse line used. This incomplete penetrance is almost certainly due to variability in the levels of Shh inactivation seen in the palatal epithelium of these mice [38]. In order to circumvent these problems, *Osr2-IresCre; Smo*^{c/c} mice have been generated, which lack *Smo* function in the palatal shelf mesenchyme from the onset of palatal development [38]. These mice have a completely penetrant cleft of the secondary palate, associated with reduced proliferation throughout the palatal shelf mesenchyme and anterior epithelium. Moreover, this proliferation

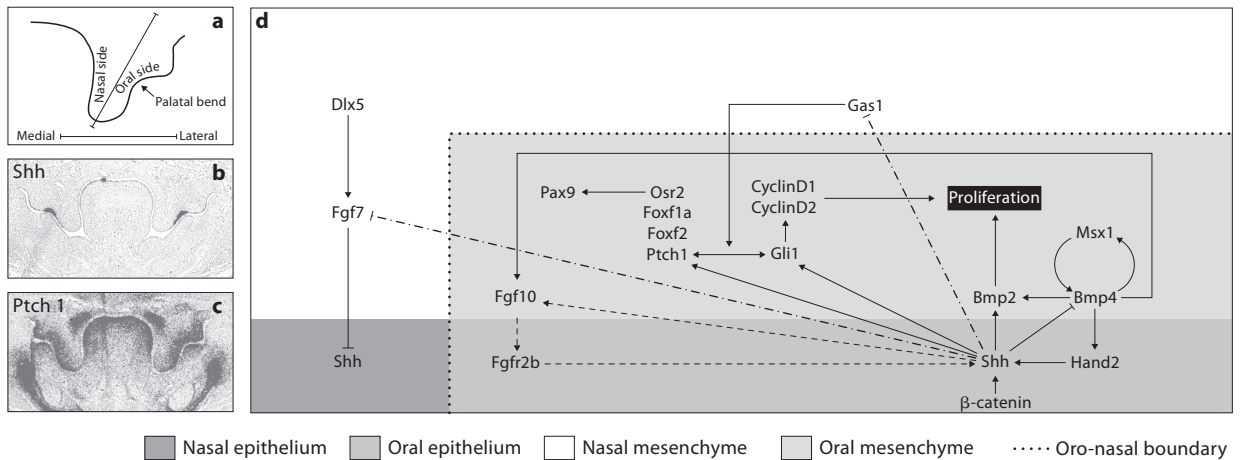


Fig. 2. Shh signalling interactions within the secondary palatal shelf at E13.5. **a** In the frontal section the palatal shelf is demarcated from the body of the maxilla by the palatal bend or anteroposterior groove (arrow) and divided into oral and nasal sides (black line). **b** *Shh* is expressed in the oral epithelium. **c** *Ptch1* expression broadly demarcates the future oral region of the shelf, extending laterally to medially within the epithelium and mesenchyme and in a gradient from the oral side to the nasal. **d** *Shh* expression is induced in the oral epithelium through *Bmp4* in the underlying mesenchyme, with *Bmp4* itself dependent upon a reciprocal signalling loop involving *Msx1* and negatively regulated by *Shh*. In addition, *Shh* is maintained in the oral epithelium via the induction of *Fgf10* and reciprocal signalling through *Fgfr2b* (---). In rugae 2 and 3, *Bmp4* is also required to maintain *Shh* expression in the epithelium through *Fgf10*. Direct transcriptional targets of *Shh* include *Ptch1* and *Gli1* within the hedgehog pathway, with *Gli1* inducing cyclinD1/2 activity and mesenchymal cell proliferation. In addition, the *Foxf1a*, *Foxf2* and *Osr2* transcription factors are also positive targets of signalling. *Gas1* is inhibited by high levels of *Shh* signal, but facilitates transduction as a co-receptor at the oral-nasal border, where *Shh* signal levels are low. A further feedback loop exists to restrict *Shh* to the oral epithelium of the palatal shelf through *Dlx5*-mediated induction of *Fgf7* on the nasal side and *Shh*-mediated repression of *Fgf7* on the oral side (---).

defect is secondary to *Shh* maintaining appropriate levels of cyclinD1 and D2 in the mesenchyme. As might be expected, *Bmp2* expression is downregulated in the anterior palate of *Osr2-IresCre; Smo^{c/c}* mice; but other positive targets of *Shh* signalling have been identified in the mesenchyme, including *Foxf1*, *Foxf2*, *Osr2* and *Fgf10*. In addition, *Bmp4* is downregulated in these mice, particularly in lateral regions of the anterior palate mesenchyme [38]. The identification of *Fgf10* as a target of *Shh* signalling establishes an important feedback loop between the epithelium and mesenchyme of the palatal shelves, seemingly required for normal cell proliferation (fig. 2). There is also evidence that *Fgf10* is partially

regulated by *Bmp4* in the anterior palate, signalling through *Bmpr1a* in the mesenchyme and contributing to maintenance of *Shh* expression in the epithelium, at least in the primary palate and rugae 2 and 3 [45]. More recently, *Shh* signalling has also been shown to be downstream of canonical Wnt signalling in the developing palate [46]. Specifically, *Shh* is lost in the palatal epithelium of *Shh^{CreGfp}; β-catenin* conditional mouse mutants, with a corresponding reduction of *Ptch1* transcription in the mesenchyme. These mice have no rugae, diminutive palatine bones, underdeveloped primary palate, spacing between the primary and secondary palates and retarded anteroposterior, but accelerated

mediolateral growth in the secondary palate; perhaps somewhat surprisingly, unassociated with changes in palatal mesenchymal cell proliferation. Moreover, tamoxifen-induced conditional ablation of *Shh* at the *Shh* locus (*Shh*-cKO) from E10.5 also results in an absence of rugae, some reduction in anteroposterior expansion, a poorly formed primary-secondary palate junction and unlike *Shh*^{Cregfp}; β -catenin mice, a cleft secondary palate [46].

The presence of graded Shh signalling within the palatal shelf mesenchyme has also suggested that thresholds of signal activity may be important for normal growth to take place. *Gas1* encodes a GPI-linked membrane glycoprotein that is repressed in response to Shh signalling, but facilitates transduction at long range in a variety of embryonic regions, acting as a co-receptor [5–7, 47, 48]. This seems to be the case during palatogenesis; *Gas1* is expressed predominantly in the nasal region of the palatal shelves, forming a gradient of transcriptional activity that is complementary to, but overlapping with *Ptch1* in the oral region. Depending upon background, *Gas1*^{-/-} mice have cleft palate with a penetrance of around 60%, associated with a reduced domain of *Ptch1* transcription within the palatal shelves and reduced proliferation in the middle and posterior mesenchyme [7]. However, overexpression of *Shh* throughout the palatal shelf epithelium in *K14-Shh* transgenic mice also reduces proliferation, in both the epithelium and mesenchyme. These mice also express *Shh* inappropriately in the medial edge epithelium at later stages of development and this prevents their fusion through an absence of apoptosis [37].

The function of Gli transcription factors has also been investigated during palatogenesis, but their collective role is not clear. Whilst *Gli1*^{-/-} mice have normal palatal development [18], both *Gli2*^{zfd} and *Gli3*^{-/-} null mutants have cleft palate [17]. In the case of *Gli2*^{zfd} mice, the cleft phenotype has around 64% penetrance and is associated with either delay or failure of palatal shelf elevation.

Although these mice have other craniofacial defects, it is likely that there is abnormal growth associated with the palatal shelves, although this has not been investigated [17]. In contrast, the cleft seen in *Gli3*^{-/-} mice does not seem to be associated with alterations in any major signalling pathways tested (*Shh*, *Bmp*, *Fgf*, *Tgf β*). Rather, the phenotype appears to be indirect, with an abnormal tongue morphology seemingly responsible for impeding normal shelf elevation and fusion [49].

The analysis of mouse mutants has demonstrated an important role for Shh signal transduction in regulating growth within the secondary palate. Pathway interruption often leads to cleft of the secondary palate, which can be secondary to severe growth retardation and an absence of shelf elevation (*K14-Shh*^{c/n}), some reduction in anteroposterior expansion (*Shh*-cKO), or relatively normal early development and successful elevation, but a failure to achieve contact in the midline, with complete (*Osr2-IresCre; Smo*^{c/c}) or more variable (*Gas1*^{-/-}) penetrance. These effects, and the molecular interactions that underlie them, suggest that Shh signalling is a key mediator of the epithelial-mesenchymal interactions that are responsible for orchestrating normal growth of the secondary palate.

Patterning along the Oro-Nasal Axis

The oro-nasal axis of the future hard palate demonstrates considerable heterogeneity, being ultimately covered by a pseudostratified ciliated columnar epithelium on the nasal side and a stratified squamous keratinized masticatory mucosa on the oral side. *Dlx5* plays an important role in patterning the oro-nasal axis of the palatal shelves, being restricted to the nasal region of the palatal shelf mesenchyme and inducing *Fgf7* expression in this region. In *Dlx5*^{-/-} mice, the domain of *Shh* expression is expanded into the nasal side of the palatal shelf because during normal development *Fgf7* acts to inhibit *Shh* in

this region of the epithelium. The consequences of this are an increased prominence of the rugae and expansion of the oral palatal epithelium, leading to the formation of a midline groove in the most severe cases [34]. These changes occur due to increased Shh-induced mesenchymal proliferation within the palatal mesenchyme of the mutant. Interestingly, Shh is also able to inhibit Fgf7, demonstrating a further feedback loop, in this case concerned with maintaining the correct spatial distribution of Shh along the palatal and nasal regions of the palatal shelf. *Dlx5*^{-/-} mice also have a shortened soft palate and ectopic uvula-like structure in this region, which results in velopharyngeal incompetence [34].

Patterning along the Anteroposterior Axis

The anteroposterior axis of the palate is clearly marked by the *Shh*-expressing palatal rugae mentioned above. Successive *Shh* stripes appear in a mid-palate anteroposterior ‘rugal growth zone’ just anterior to ruga 8 [31] where there is increased spacing between pre-existing stripes [30, 31]. The correlation between growth and new stripe appearance is maintained during development: not only do most new stripes appear in the rugal growth zone but the ‘out of sequence’ appearance of the stripe for ruga 1 is associated with medial ‘filling in’ growth at the extreme anterior of the secondary palate. The latter provides tissue at the right distance anterior to ruga 2 in which *Shh* expression can appear [50]. The correlation between growth and stripe appearance is also observed in evolution: related rodents have numbers of rugae correlated to the length of time the palate grows [30]. Moreover, it has been suggested by analogy with well-studied spacing mechanisms for teeth, and hair and feather follicles in skin, that this regular spacing might be governed by a reaction-diffusion mechanism [30]. Reaction-diffusion mechanisms, sometimes called local activation-lateral inhibition

(LALI) or Turing mechanisms, involve two diffusible morphogens, an activator and an inhibitor [51]. If the activator activates itself as well as the inhibitor, while the inhibitor inhibits both and diffuses more quickly, a regular pattern of spots or stripes can emerge. This scheme was first proposed by the mathematician Alan Turing as the simplest possible pattern-generating system [52]. In the skin it is thought that as many as six morphogens, three activating and three inhibiting are involved [53], but hitherto there have been no other convincing demonstrations that this elegant mechanism applies in vivo. In the palate, loss of Fgf10, Fgfr2 or β -catenin leads to loss of rugae [42, 46] implicating FGF and canonical Wnt signalling as activating morphogens in this system. Inhibitor experiments on palatal explants and epithelial-specific knock-down of Shh signalling implicate Shh as an inhibitor. Specifically, in explant studies, the Shh-inhibitor cyclopamine upregulates rugal stripe markers while purmorphamine, an Shh-mimic, suppresses them. Excision of a stripe causes neighbouring stripes to branch consistent with a simple reaction diffusion mechanism [50]. However, given the complexities of skin and tooth patterning, it is likely that additional morphogens will be found. Multiple morphogens, including *Shh*, working in a complex network with multiple layers of feedback are thought to provide robustness and coordination with other processes necessary for reliable development [54]. The beautifully rectilinear and iterative pattern of the rugae suggest the palate as an excellent model system for unravelling these complexities.

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References

- Ingham PW, McMahon AP: Hedgehog signaling in animal development: paradigms and principles. *Genes Dev* 2001;15:3059–3087.
- Ingham PW, Nakano Y, Seger C: Mechanisms and functions of Hedgehog signalling across the metazoa. *Nat Rev Genet* 2011;12:393–406.
- Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA: Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* 1996;383:407–413.
- Goodrich LV, Johnson RL, Milenkovic L, McMahon JA, Scott MP: Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev* 1996;10:301–312.
- Allen BL, Tenzen T, McMahon AP: The Hedgehog-binding proteins Gas1 and Cdo cooperate to positively regulate Shh signaling during mouse development. *Genes Dev* 2007;21:1244–1257.
- Martinelli DC, Fan CM: Gas1 extends the range of Hedgehog action by facilitating its signaling. *Genes Dev* 2007;21:1231–1243.
- Seppala M, Depew MJ, Martinelli DC, Fan CM, Sharpe PT, Cobourne MT: Gas1 is a modifier for holoprosencephaly and genetically interacts with Sonic hedgehog. *J Clin Invest* 2007;117:1575–1584.
- Tenzen T, Allen BL, Cole F, Kang JS, Krauss RS, McMahon AP: The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice. *Dev Cell* 2006;10:647–656.
- Stone DM, Hynes M, Armanini M, Swanson TA, Gu Q, Johnson RL, Scott MP, Pennica D, Goddard A, Phillips H, Noll M, Hooper JE, de Sauvage F, Rosenthal A: The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* 1996;384:129–134.
- Taipale J, Cooper MK, Maiti T, Beachy PA: Patched acts catalytically to suppress the activity of Smoothened. *Nature* 2002;418:892–897.
- Zhang XM, Ramalho-Santos M, McMahon AP: Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. *Cell* 2001;105:781–792.
- Casali A, Struhl G: Reading the Hedgehog morphogen gradient by measuring the ratio of bound to unbound Patched protein. *Nature* 2004;431:76–80.
- Chen Y, Struhl G: Dual roles for patched in sequestering and transducing Hedgehog. *Cell* 1996;87:553–563.
- Dessaud E, Yang LL, Hill K, Cox B, Ulloa F, Ribeiro A, Mynett A, Novitsch BG, Briscoe J: Interpretation of the Sonic hedgehog morphogen gradient by a temporal adaptation mechanism. *Nature* 2007;450:717–720.
- Bai CB, Auerbach W, Lee JS, Stephen D, Joyner AL: Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. *Development* 2002;129:4753–4761.
- Bai CB, Stephen D, Joyner AL: All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3. *Dev Cell* 2004;6:103–115.
- Mo R, Freer AM, Zinyk DL, Crackower MA, Michaud J, Heng HH, Chik KW, Shi XM, Tsui LC, Cheng SH, Joyner AL, Hui C: Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development* 1997;124:113–123.
- Park HL, Bai C, Platt KA, Matise MP, Beeghly A, Hui CC, Nakashima M, Joyner AL: Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. *Development* 2000;127:1593–1605.
- Stamatiki D, Ulloa F, Tsoni SV, Mynett A, Briscoe J: A gradient of Gli activity mediates graded Sonic hedgehog signaling in the neural tube. *Genes Dev* 2005;19:626–641.
- Pan Y, Bai CB, Joyner AL, Wang B: Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. *Mol Cell Biol* 2006;26:3365–3377.
- Tempé D, Casas M, Karaz S, Blanchet-Tournier MF, Concordet JP: Multisite protein kinase A and glycogen synthase kinase 3 β -phosphorylation leads to Gli3 ubiquitination by SCF β TrCP. *Mol Cell Biol* 2006;26:4316–4326.
- Wang B, Li Y: Evidence for the direct involvement of β TrCP in Gli3 protein processing. *Proc Natl Acad Sci USA* 2006;103:33–38.
- Huangfu D, Anderson KV: Cilia and Hedgehog responsiveness in the mouse. *Proc Natl Acad Sci USA* 2005;102:11325–11330.
- Huangfu D, Liu A, Rakeman AS, Murcia NS, Niswander L, Anderson KV: Hedgehog signalling in the mouse requires intraflagellar transport proteins. *Nature* 2003;426:83–87.
- Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DY, Reiter JF: Vertebrate Smoothened functions at the primary cilium. *Nature* 2005;437:1018–1021.
- Haycraft CJ, Banizs B, Aydin-Son Y, Zhang Q, Michaud EJ, Yoder BK: Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. *PLoS Genet* 2005;1:e53.
- Liu A, Wang B, Niswander LA: Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors. *Development* 2005;132:3103–3111.
- May SR, Ashique AM, Karlen M, Wang B, Shen Y, Zarbalis K, Reiter J, Ericson J, Peterson AS: Loss of the retrograde motor for IFT disrupts localization of Smo to cilia and prevents the expression of both activator and repressor functions of Gli. *Dev Biol* 2005;287:378–389.
- Rohatgi R, Milenkovic L, Scott MP: Patched1 regulates hedgehog signaling at the primary cilium. *Science* 2007;317:372–376.
- Pantalacci S, Prochazka J, Martin A, Rothova M, Lambert A, Bernard L, Charles C, Viriot L, Peterkova R, Laudet V: Patterning of palatal rugae through sequential addition reveals an anterior/posterior boundary in palatal development. *BMC Dev Biol* 2008;8:116.

- 31 Welsh IC, O'Brien TP: Signaling integration in the rugae growth zone directs sequential SHH signaling center formation during the rostral outgrowth of the palate. *Dev Biol* 2009;336:53–67.
- 32 Xiong W, He F, Morikawa Y, Yu X, Zhang Z, Lan Y, Jiang R, Cserjesi P, Chen Y: Hand2 is required in the epithelium for palatogenesis in mice. *Dev Biol* 2009;330:131–141.
- 33 He F, Xiong W, Wang Y, Li L, Liu C, Yamagami T, Taketo MM, Zhou C, Chen Y: Epithelial Wnt/ β -catenin signaling regulates palatal shelf fusion through regulation of Tgfb3 expression. *Dev Biol* 2011;350:511–519.
- 34 Han J, Mayo J, Xu X, Li J, Bringas P Jr, Maas RL, Rubenstein JL, Chai Y: Indirect modulation of Shh signaling by Dlx5 affects the oral-nasal patterning of palate and rescues cleft palate in Msx1-null mice. *Development* 2009;136:4225–4233.
- 35 Sagai T, Amano T, Tamura M, Mizushina Y, Sumiyama K, Shiroishi T: A cluster of three long-range enhancers directs regional Shh expression in the epithelial linings. *Development* 2009;136:1665–1674.
- 36 Rice R, Connor E, Rice DP: Expression patterns of Hedgehog signalling pathway members during mouse palate development. *Gene Expr Patterns* 2006;6:206–212.
- 37 Cobourne MT, Xavier GM, Depew M, Hagan L, Sealby J, Webster Z, Sharpe PT: Sonic hedgehog signalling inhibits palatogenesis and arrests tooth development in a mouse model of the nevroid basal cell carcinoma syndrome. *Dev Biol* 2009;331:38–49.
- 38 Lan Y, Jiang R: Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth. *Development* 2009;136:1387–1396.
- 39 Yamagishi C, Yamagishi H, Maeda J, Tsuchihashi T, Ivey K, Hu T, Srivastava D: Sonic hedgehog is essential for first pharyngeal arch development. *Pediatr Res* 2006;59:349–354.
- 40 Jeong J, Mao J, Tenzen T, Kottmann AH, McMahon AP: Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. *Genes Dev* 2004;18:937–951.
- 41 Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen Y: Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. *Development* 2002;129:4135–4146.
- 42 Rice R, Spencer-Dene B, Connor EC, Gritli-Linde A, McMahon AP, Dickson C, Thesleff I, Rice DP: Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. *J Clin Invest* 2004;113:1692–1700.
- 43 Dassule HR, Lewis P, Bei M, Maas R, McMahon AP: Sonic hedgehog regulates growth and morphogenesis of the tooth. *Development* 2000;127:4775–4785.
- 44 Gritli-Linde A, Bei M, Maas R, Zhang XM, Linde A, McMahon AP: Shh signaling within the dental epithelium is necessary for cell proliferation, growth and polarization. *Development* 2002;129:5323–5337.
- 45 Baek JA, Lan Y, Liu H, Maltby KM, Mishina Y, Jiang R: Bmpr1a signaling plays critical roles in palatal shelf growth and palatal bone formation. *Dev Biol* 2011;350:520–531.
- 46 Lin C, Fisher AV, Yin Y, Maruyama T, Veith GM, Dhandha M, Huang GJ, Hsu W, Ma L: The inductive role of Wnt- β -catenin signaling in the formation of oral apparatus. *Dev Biol* 2011;356:40–50.
- 47 Allen BL, Song JY, Izzi L, Althaus IW, Kang JS, Charron F, Krauss RS, McMahon AP: Overlapping roles and collective requirement for the coreceptors GAS1, CDO, and BOC in SHH pathway function. *Dev Cell* 2011;20:775–787.
- 48 Izzi L, Levesque M, Morin S, Laniel D, Wilkes BC, Mille F, Krauss RS, McMahon AP, Allen BL, Charron F: Boc and Gas1 each form distinct Shh receptor complexes with Ptch1 and are required for Shh-mediated cell proliferation. *Dev Cell* 2011;20:788–801.
- 49 Huang X, Goudy SL, Ketova T, Litingtung Y, Chiang C: Gli3-deficient mice exhibit cleft palate associated with abnormal tongue development. *Dev Dyn* 2008;237:3079–3087.
- 50 Economou AD, Ohazama A, Porntavee T, Sharpe PT, Kondo S, Basson MA, Gritli-Linde A, Cobourne MT, Green JBA: Periodic stripe formation by a Turing mechanism operating at growth zones in the mammalian palate. *Nat Genet* 2012;44:348–351.
- 51 Kondo S, Miura T: Reaction-diffusion model as a framework for understanding biological pattern formation. *Science* 2010;329:1616–1620.
- 52 Turing AM: The chemical nature of morphogenesis: A reaction-diffusion model for development. *Phil Trans R Soc Lond* 1952;237:37–72.
- 53 Headon DJ, Palmer KJ: Stippling the skin: Generation of anatomical periodicity by reaction-diffusion mechanisms. *Math Model Nat Phenom* 2009;4:83–102.
- 54 Lander AD, Nie Q, Wan FY: Do morphogen gradients arise by diffusion? *Dev Cell* 2002;2:785–796.

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Roles of BMP Signaling Pathway in Lip and Palate Development

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Abstract

Cleft lip with or without cleft palate (CLP) and cleft palate only (CP) are severe disruptions affecting orofacial structures. Patients with orofacial clefts require complex interdisciplinary care, which includes nursing, plastic surgery, maxillofacial surgery, otolaryngology, speech therapy, audiology, psychological and genetic counseling, orthodontics and dental treatment, among others. Overall, treatment of clefts of the lip and palate entails a significant economic burden for families and society. Therefore, prevention is the ultimate objective and this will be facilitated by a complete understanding of the etiology of this condition. Here we review the current concepts regarding the genetic and environmental factors contributing to orofacial clefts and emphasize on the roles of BMP signaling pathway components in the normal and aberrant development of the lip and palate.

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Epidemiology and Genetics of CLP and CP

Orofacial clefts affecting lip and palate may be classified as: (1) complete cleft palate with cleft lip; (2) cleft of the primary (anterior) palate, in which the cleft is limited to the anterior incisive fossa, and may or may not involve cleft lip; (3) cleft of the secondary (posterior) palate, in

which the cleft defect is limited to the posterior incisive fossa and (4) submucosal cleft including bifid uvula [1–7]. CLP and CP can be isolated features (non-syndromic) or part of a syndrome (syndromic). Approximately 70% of all cases of CLP and 50% of cases of CP are considered to be non-syndromic. Non-syndromic cases of CLP and CP occur with a high prevalence in diverse human populations. Non-syndromic CLP affects approximately 1 in 700 live births, with large variability in populations depending on the ethnic group, geographic origin as well as the environmental exposures and social and economic conditions. Asian and Native American populations have the highest prevalence, as high as 1 in 500. Caucasian populations have an intermediate prevalence of 1 in 1,000, and African-derived populations have the lowest prevalence at 1 in 2,500. The occurrence of CLP and CP also changes by gender and laterality of the cleft with males being more affected than females by clefts involving the lip at a 2:1 male to female ratio. On the other hand, the ratio for clefts affecting only the palate is inverse, a 1:2 male to female. Among unilateral cases of CLP, a 2:1 ratio of left- to right-sided clefts has been reported [8].

The etiology of CLP and CP in humans is heterogeneous, involving genetic and environmental factors. Orofacial clefts have an elevated rate of familial recurrence compared with other birth defects. It has been reported that the risk of cleft recurrence in first-degree relatives is 32 for cleft lip (CL) and 56 for CP compared to the reference populations, which suggest a stronger genetic background for CP compared with CL [9]. In studies involving twin pairs, the concordance rate of 40–60% in monozygotic (MZ) twins is significantly higher than that observed in dizygotic (DZ) twins, which is only 3–5%. The high concordance rate in MZ twins provides substantial evidence supporting a strong genetic component [10]. However, the fact that few pedigrees of patients affected by CLP or CP show mendelian inheritance and most cases appear to be sporadic rules out a purely genetic or purely environmental cause for orofacial clefting. In fact, environmental risk factors have been reported to be an important component of the CLP and CP etiology [11].

A multifactorial model in which multiple and probably small genetic risk factors might interact with environmental agents has been suggested [8, 11]. The identification of individual genetic factors for clefting has been a goal of numerous research groups since the first reported association between CLP and gene variants of the Transforming Growth Factor Alpha (TGFA) by Ardinger et al. [12]. From this study, the list of candidate genes for CLP and CP has increased quickly. Currently, this list includes IRF6, MSX1, TGFB3, ABCA4, FOXE1, FGFR1, FGFR2, FGF8, MAFB, PDGFC, CRISPLD2, PVRL1, GABRB3, MSX2, SATB2, TBX10, TBX22, GLI2, JAG2, MTHFR, RARA, LHX8, SKI and SPRY2, among others [8, 10, 13–20]. Methods to identify those genes include association studies using case-parent trios or case-control samples; identification of chromosomal anomalies or microdeletions in cases, and direct sequencing of DNA samples from affected individuals as well as genome-wide strategies [8]. There have also been several resequencing studies

of candidate genes to identify specific variants that may possess statistical associations with orofacial clefting. The strongest existing data is for mutations in *MSX1* [21], *FGFR1* and *FGF8* [19], and bone morphogenetic protein 4 (*BMP4*) [22].

An environmental component to clefting has been also recognized, especially in regions of low socioeconomic status. Among the environmental factors contributing to CLP and CP, maternal exposure to certain drugs such as phenytoin, valproic acid, retinoids and thalidomide has been reported [23]. Increased risk of CLP and CP has been connected to maternal alcohol or cigarette use [24, 25], herbicides such as dioxin [26], altitude [27], hyperthermia [28] and infectious diseases [29]. Specifically, maternal smoking appears to contribute to 4% of the total CLP cases and 12% of bilateral CLP [30]. Several meta-analyses support a significant interaction between maternal smoking and CLP [31, 32]. However, a meta-analysis of five published studies demonstrated that maternal smoking and infant TGFA genotype are associated with CP but not with CLP [33]. Smoking has also been associated with variants of Glutathione S-transferase-01 (*GSTT1*), Nitric Oxide Synthase 3 (*NOS3*) and *IRF6* in the etiology of CLP [8, 34]. Other studies also support interactions between smoking, alcohol consumption, nutritional factors and the *MSX1* and *TGFB3* genes in addition to TGFA [reviewed in 23]. Regarding alcohol exposure, among the anomalies characterizing fetal alcohol syndrome are the alterations of the craniofacial region, including orofacial clefts. The specific association between CLP and CP with alcohol consumption has also been suggested from case-control studies but it is more inconsistent compared to maternal smoking [8]. Some studies indicate that high doses of alcohol in short periods of time increase the risk [35] and others suggest an association of polymorphic variants of the Alcohol Dehydrogenase 1C (*ADH1C*) gene and CLP [36]. More research is needed to understand the complex gene-environment interactions leading to CLP and CP.

Lip and Palate Development

Although CLP is the most frequent type of cleft in humans, most mouse models for clefting display CP only. The upper lip and nose form before the fusion of the secondary palatal shelves [37]. A failure of correct lip fusion may secondarily have an effect on palatal fusion, which explains why CP accompanies clefts of the lip. At embryonic day 10.5 (E10.5) in the mouse and in the fifth week of pregnancy in humans, the surface ectoderm of the frontonasal prominence thickens developing the nasal placodes. Next, the lateral and medial nasal processes emerge from the prominence around the placode. The nasal pit is established in the center. The maxillary processes of the first branchial arch grow towards the medial and lateral nasal processes at E10.5 in mice and the 35th day in humans and together form the upper lip. The fusion processes between the medial and lateral nasal prominences begin at this stage. Subsequently, the maxillary and medial processes also undergo fusion. At E12.5 in mouse and seventh week in humans, the formation of the upper lip is finished after complete disappearance of the epithelial seams between the processes [37].

The palate in mammals forms from two primordia: the primary and the secondary palate. The primary palate, which shares embryological origin with the lip, represents only a small part of the adult hard palate. The development of the secondary palate begins at E12.5 in mice and the sixth week in humans with the emergence of palatal primordia from the maxillary process [38]. The palatal shelves are composed of cranial neural crest-derived (CNCC) mesenchyme covered by pharyngeal ectoderm-derived epithelium [38, 39]. Using the *Wnt1-Cre;R26R* model, we have shown that more than 90% of palatal mesenchymal cells are derived from CNCC [39, 40]. From E12.5 to E13.5, the palatal shelves grow vertically along the two sides of the tongue. Once the mandible starts growing in length providing physical space for the tongue to descend, the palatal

shelves reorient to acquire a horizontal position. Then, during E14.5–15.5 in mice and week 9 in humans, the palatal shelves grow toward each other and establish contact in the midline, where a midline epithelial seam is formed (MES) [41]. The MES must degenerate in order for the mesenchyme to become continuous and several theories have been proposed for this process: programmed cell death [42], migration of the epithelial cells to the oral or nasal side of the palate [43], and/or epithelial-mesenchymal transformation (EMT) [44]. However, epithelial cell mapping using the *K14-Cre;R26R* model shows that midline epithelial cells mainly disappear due to apoptosis and migration, but not EMT [45]. By E16.5 in mice and the 12th week in humans, fusion of the palatal shelves is complete. Next, ossification occurs in the anterior two-thirds of the palate to form the hard palate whereas the posterior third develops into the soft palate without ossification [reviewed in 46]. The nasal and oral epithelia at this stage are differentiated pseudostratified and squamous epithelia, respectively [reviewed in 7].

Lip and palate development rely on a very tightly regulated network that includes the activity of secreted proteins and their signaling pathways, cell-surface receptors, ECM components as well as transcription factors [reviewed in 46]. The transforming growth factor-beta (TGF β) superfamily is an important part of this network as deficiency of its members leads to malformations affecting the craniofacial region and particularly the palate in mouse models. The TGF β superfamily includes activin, TGF β and BMP cytokines [47].

BMP Signaling Pathway

BMPs are secreted signaling molecules that trigger intracellular cascades by binding to cell surface receptor serine/threonine kinases [48]. More than 20 members have been identified in the BMP family [49]. BMP ligands are classified into five subfamilies based on phylogenetic studies

and sequence similarity: (1) the *dpp* subfamily is comprised of BMP2 and BMP4 because of their similarity to the *Drosophila dpp* gene (decapentaplegic); (2) the 60A subfamily includes BMP5, -6, -7 and -8; (3) BMP3 and BMP3b (GDF10) together constitute a unique subfamily; (4) GDF5, -6, -7 constitute another subgroup in this family, and (5) Nodal and Lefty are distant members of the BMP subfamily [reviewed in 50, 51]. BMPs mainly signal through BMP receptors (BMPR) type I and type II. Three type I (ALK2, ALK3 or BMPRIA and ALK6 or BMPRIb) and three type II receptors (BMPRII, ActRIIA and ActRIIB) are known to mediate BMP signals [52]. Upon ligand binding, the type II receptor phosphorylates the type I receptor. In turn, type I receptor phosphorylates and activates a group of transcriptional coactivators called Smads, specifically Smad1/5/8. These Smads associate with Smad4 (common Smad) forming a complex that is able to translocate into the nucleus where it regulates the transcription of downstream targets, including *Msx* genes [reviewed in 50]. Vertebrate *Msx* genes are unlinked, homeobox-containing genes that are homologous to the *Drosophila muscle segment homeobox* gene. They are regulatory proteins that function as transcriptional repressors in vitro and in vivo. *Msx* proteins also interact with other homeodomain proteins to regulate transcription. In fact, heterodimers formed between *Msx1* and other homeodomain proteins such as *Dlx2*, *Dlx5*, *Lhx2* and *Pax3* appear to result in mutual functional antagonism [reviewed in 53]. *Msx* genes are considered readouts of the BMP signaling pathway (see below).

Fine-tuning of the BMP pathway is primarily achieved through the action of antagonists, co-receptors and intracellular regulatory proteins. BMP antagonists are secreted peptides that bind to BMP ligands, blocking their receptor epitopes, and consequently prevent ligand-receptor oligomerization. BMP antagonists are structurally similar to BMP ligands as they are homodimeric proteins that consist of a cystine knot motif. BMP

antagonists include: the Chordin/Noggin family, Twisted Gastrulation (Tsg) and the DAN/Cerberus family [51, 54]. On the other hand, BMP signaling can be modulated by co-receptors. Repulsive guidance molecules (RGM) a and c, Dragon (RGMb), c-Kit, Endoglin and Betaglycan are co-receptors that enhance BMP signal transduction [55, 56]. The decoy-receptor BMP and activin membrane-bound protein (BAMBI), Ror2 and TrkC receptors regulate BMP signaling negatively [57, 58]. Finally, there is a set of intracellular proteins that act as signal transducers or regulators of BMP signaling. Among them, BMPRIA interacts with the TGF β -activated kinase (TAK) binding protein 1 (TAB1) through the BMP receptor-associated molecule 1 (BRAM1), which downregulates BMP-mediated Smad signaling [59]. Likewise, the X-chromosome-linked inhibitor of apoptosis (XIAP) connects BMPRI with TAB1 and may act as a positive regulator of MAP kinase signaling [60]. In addition to proteins that regulate the MAP kinase pathway, BMPRI also interacts with membrane endocytic factors (caveolin1, Eps15R), phosphatases (Dullard, PP2A), kinases (Rack1) and cytoskeletal proteins (Trb3), among others (for more details on BMP modulation, see Sieber et al. [51]).

BMP Signaling Pathway in Palate Development

Expression Patterns

The BMP signaling pathway regulates a wide array of developmental processes such as cell proliferation, apoptosis, differentiation, and morphogenesis [reviewed in 61]. *Bmp2* and *Bmp4* mRNAs are expressed in a dynamic pattern in the facial primordia of chick and mammal embryos. *Bmp4* expression is highly restricted to the distal epithelia of the medial nasal, lateral nasal, maxillary and mandibular processes [62]. The expression patterns of *Bmp2* and *Bmp4* in the facial ectoderm correlate with the underlying

mesenchymal expression domains of *Msx1* and *Msx2* in the facial primordia. Furthermore, ectopic Bmp2 or Bmp4 is able to induce *Msx1* and *Msx2* gene expression in the facial mesenchyme [63]. This finding suggests that *Msx1* and *Msx2* are downstream targets of the Bmp pathway. In fact, a BMP-responsive region of *Msx2* has been recently identified in mammalian cells [64]. *Bmp4* expression has also been detected in the distal ectoderm of the facial primordia neighboring the stomodeum prior to and during lip fusion in mouse embryos [65]. Accordingly, *Msx1* and *Msx2* expression is detectable in the adjacent facial mesenchyme.

In the mouse developing palate, *Bmp4* mRNA is found in the palatal epithelium and mesenchyme at E12.5 and it is subsequently restricted to the mesenchyme at E13.5 and E14.5. *Bmp4* expression is absent in the posterior region of E13.5 and E14.5 palatal shelves. *Bmp2* presents a similar pattern of expression. At E12.5 and E13.5 it is expressed in both the epithelium and mesenchyme of the anterior region of developing palatal shelves and is absent in the posterior palate [66]. Reports of a weak, diffuse expression of *Msx1* in the palatal mesenchyme provided the first evidence that *Msx1* may have a direct role in palate development. A more detailed analysis by Zhang et al. [66] has reported that *Msx1* expression in the palatal mesenchyme is confined to the anterior portion of the developing palatal shelves. Recently, it has been shown that phospho-Smad1/5/8, which marks the activation of BMP signaling, appears to be restricted to the nasal side of the palatal mesenchyme although the expression of *Bmp4* and *Bmp2* is homogeneous throughout the palatal mesenchyme [66, 67]. The expression pattern of *Bmpr1a* and other receptors in the palatal mesenchyme needs to be examined to explain the asymmetrical activation of BMP signaling in the palatal mesenchyme. This asymmetry might be due to (1) regional differential expression of *Bmpr*, which determines the establishment of the BMP-responsive domain

in the palatal mesenchyme and/or (2) inhibition of BMP signaling activation on the oral side of the palatal mesenchyme.

Animal Models

The first functional analyses of BMP and its downstream targets were performed in chick embryos. For instance, ectopic application of Bmp2 or Bmp4 protein induces overgrowth and changes the patterning of the chick facial prominences [63]. Conversely, inhibiting Bmp signaling by application of Noggin in the chick facial primordia causes reduced mesenchymal proliferation and outgrowth [68, 69]. More recently, Bmp signaling has been suggested to be crucial in the evolution of facial shape and size in fish and birds [reviewed in 70]. Analyses of mouse models have provided information regarding the mechanisms that BMP uses to exert its functions in palatogenesis.

The direct study of Bmp signaling pathway components in craniofacial development has been hampered by the early embryonic lethality of the *Bmpr*-null mutant mice and functional redundancy among BMP ligands [71–73]. Liu et al. [74] used conditional null alleles of *Bmpr1a* and *Bmp4* to overcome the early embryonic lethality of conventional *Bmpr1*- and *Bmp4*-null mice. *Nestin-Cre;Bmpr1a* mutants display bilateral CLP and arrested tooth formation with complete penetrance. The cleft palate of *Nestin-Cre;Bmpr1a^{fl/fl}* embryos is associated with reduced mesenchymal cell proliferation in the maxillary process and defective anterior-posterior patterning. In contrast, there is increased apoptosis in the fusing region of the medial nasal process in *Nestin-Cre;Bmpr1a^{fl/fl}* mice. Moreover, conditional inactivation of the *Bmp4* gene using the *Nestin-Cre* transgenic line results in isolated cleft lip. These findings contrast with previous results in which inhibition of BMP signaling in the chick facial primordia with Noggin increased epithelial survival [68], suggesting that the epithelial function of BMP during craniofacial development is

still not completely understood. Liu et al. [74] also reported that numerous mouse embryos in which *Bmp4* is inactive in facial epithelium have delayed lip fusion that, however, is repaired by E14.5 in most mutant mice. It is hypothesized that the absence of cleft lip in these mutants is due to functional redundancy with other members of the *Bmp* family. Interestingly, specific inactivation of *Bmpr1a* in CNCC-derived mesenchyme from E10.5 (*Osr2-Cre;Bmpr1a^{fl/fl}*) causes reduced cell proliferation in the primary and anterior secondary palate, which result in partial cleft of the anterior palate. In these mutants, *Msx1* and *Fgf10* expression is downregulated in the anterior palate mesenchyme and expression of *Shh* is reduced in the overlying palatal epithelium. These findings indicate that Bmp signaling regulates mesenchymal-epithelial interactions during palatal outgrowth. Additionally, the development of the palatal processes of the maxilla is deficient and formation of the palatal processes of the palatine is significantly delayed, which lead to a submucous cleft of the hard palate in *Osr2-Cre;Bmpr1a^{fl/fl}* mice [75]. Similarly, mice lacking *Alk2* in the neural crest (*Wnt1-Cre;Alk2^{fl/fl}*) display multiple craniofacial defects including CP and a hypotrophic mandible [76].

Conventional knockout mice for downstream elements of the BMP pathway have been also generated. In the case of *Smad4*, the early embryonic lethality in *Smad4*-null mice makes analysis of its effects on craniofacial structures impossible [77, 78]. Inactivation of *Smad4* in the palatal epithelium using *K14-Cre* does not lead to any defect in palate development. However, this result is due to functional redundancy between *Smad4*-dependent and -independent pathways, specifically p38 MAPK [79], and needs a careful interpretation because *Smad4* acts as a common Smad for both TGF β and BMP signaling pathways. Inactivation of *Smad4* in CNCC-derived mesenchyme, in *Wnt1-Cre;Smad4^{fl/fl}* mice, shows that *Smad4* is not required for the migration of CNCC although embryonic development is arrested at

E11.5–E12.5. There is underdevelopment of the first branchial arch and failure of fusion in the middle of the frontonasal process and in the middle of the mandibular process of the first branchial arch in *Wnt1-Cre;Smad4^{fl/fl}* embryos. The defects in lateral development of the first branchial arch are more severe than those in anterior-posterior development due to a dramatic increase in the number of apoptotic cells in the CNCC mesenchyme of *Wnt1-Cre;Smad4^{fl/fl}* mice [80]. The analysis of *Smad4* function during palatogenesis in these conditional mutant embryos is impeded due to the early arrested development. To overcome this issue, *Osr2-Cre;Smad4* embryos have been generated. These mice display complete CP due to a significant reduction in mesenchymal cell proliferation, which occurs from E14.5 [pers. unpubl. data].

Satokata and Mass [81] generated *Msx1*-deficient mice and neonatal mortality, CP and anodontia are their main phenotypic features. Mice lacking both *Msx1* and *Msx2* gene function exhibit bilateral CLP [82]. *Msx1* and *Msx2* play critical roles in facial mesenchymal proliferation because *Msx1*-null mutant mice have shortened maxilla and mandible as well as defects in palatal mesenchyme proliferation [66, 81]. Using a transgenic approach, Zhang et al. [66] expressed a *Bmp4* transgene ectopically in the *Msx1* mutant palatal mesenchyme, resulting in rescue of the cleft palate phenotype and neonatal lethality. Associated with this rescue was a restored pattern of *Shh* and *Bmp2* expression, as well as a recuperation of normal cell proliferation in the palatal mesenchyme. Therefore, BMP4 appears to avoid a requirement for *Msx1* and to function upstream of *Shh* and *Bmp2* to regulate palatogenesis [66]. In addition, *Shh* derived from the midline epithelial cells is able to activate *Bmp2* expression in the mesenchyme, which in turn stimulates cell proliferation. Thus, *Msx1* controls a genetic hierarchy involving BMP and *Shh* signals regulating the growth of the anterior palate during development.

The role of *Msx1* in the palate has been further analyzed by generating double (*Msx1*^{-/-};*Dlx5*^{-/-}) conventional knockout mice [67, 83]. Inactivation of *Dlx5* significantly stimulates palatal mesenchymal proliferation in the background of *Msx1*-null mutation, although proliferation activity is not altered in *Dlx5*-null single mutants [67]. Rescue of cell proliferation is linked with a complete rescue of the *Msx1*^{-/-} cleft palate defect in *Msx1*^{-/-};*Dlx5*^{-/-} mutant mice. The fact that *Dlx5* expression is not compromised in *Msx1*-null palates suggests an indirect relationship between these two genes in palatogenesis as well as a complex mechanism leading to the rescue of *Msx1*-null phenotype. Interestingly, in *Dlx5*-null embryos, the oral side of the palatal shelves is expanded. This finding is based on the expansion of *Shh* expression into the midline epithelium. This is an expansion, not a shift, as the oral side *Shh* expression persists. The change in *Shh* expression in *Dlx5*-null mice is accompanied by an expansion of *Gli1* in the palatal mesenchyme from the oral region into the nasal portion and a reduction of nasal markers such as Phospho-Smad1/5/8 and *Fgf7* [67]. Previous studies have shown that increased *Shh* signaling has a stimulatory role on cell proliferation in the palatal mesenchyme [66]. Accordingly, *Shh* expansion is responsible for the rescue of cell proliferation in the *Msx1*^{-/-};*Dlx5*^{-/-} palates [67]. Moreover, *Dlx5* is required for *Fgf7* expression and FGF7 inhibits the expression of *Shh* in the palatal epithelium.

Regarding BMP modulators, previous studies have shown that loss of *Noggin* function in palatal epithelial cells leads to overactive BMP signaling, particularly in the palatal epithelium. This results in alteration of cell proliferation and excessive cell death leading to complete CP. The integrity of the palatal epithelium is compromised due to the excessive cell death, which in turn leads to abnormal palate-mandible fusion and blocks palatal shelf reorientation. This phenotype is also observed in ectopic expression of a constitutively active form of BMPR-IA but not BMPR-IB in the palatal epithelium [84]. These findings underscore the

importance of modulation of BMP signaling during palatogenesis.

BMP and Orofacial Clefts in Humans

Suzuki et al. [22] have identified missense and nonsense mutations in the BMP4 gene in 1 of 30 cases of microform clefts, 2 of 87 cases with subepithelial defects in the orbicularis oris muscle, 5 of 968 cases of overt CLP, and 0 of 529 controls. These results provide confirmation that microforms and subepithelial orbicularis oris muscle defects are part of the spectrum of CLP. Heterozygous variations in BMP7, including a frame shift, missense, or Kozak sequence mutation, in individuals with developmental eye anomalies and a range of systemic abnormalities, including developmental delay, deafness, scoliosis, and CP have also been reported [85]. Historically, MSX1 has been the subject of human geneticists since at least the early 1990s. In 1997, Lidral et al. [86] proposed a possible role for MSX1 in non-syndromic CLP but they did not detect disease-linked mutations. In addition, some studies have supported an interaction between environmental factors and MSX1 [21]. For instance, the risk of CLP and CP related to maternal cigarette smoking and alcohol consumption during pregnancy, increases due to the interaction of such exposure and specific polymorphic variants at the MSX1 gene [86]. Interestingly, MSX1 mutation was observed in patients with tooth agenesis and CLP or CP only [21]. Since anodontia and hypodontia have been observed in patients with CP it may be possible that MSX1 correlates with a syndromic form of cleft including tooth agenesis. Thus, MSX1 may be involved in syndromic and non-syndromic CLP and CP. Jezewski et al. [87] identified a mutation in the MSX1 gene in patients with CLP in Filipinas. Suzuki et al. [88] and Vieira et al. [89] detected a missense mutation in the MSX1 gene in Vietnamese and Chilean CLP patients, respectively. More recently, positive association between MSX1 gene variants and non-syndromic CLP was found in South Indian patients [90], Malay population [91], Han Chinese

in Western China [92], Estonian population [93] and Colombian populations [94], among multiple studies in diverse populations.

Concluding Remarks

Numerous studies in animal models and humans have shown that BMP signals exert diverse and crucial functions during craniofacial development

and particularly during palatogenesis. More research is needed to elucidate specifically how BMP signaling regulates different cellular activities, what downstream targets are activated and how it interacts with other signaling pathways during lip and palate development. This information will provide us with a comprehensive understanding of this pathway and with the opportunity of therapeutic approaches for malformations of the lip and palate in humans.

References

- Aminpour S, Tollefson TT: Recent advances in presurgical molding in cleft lip and palate. *Curr Opin Otolaryngol Head Neck Surg* 2008;16:339–346.
- Kasten EF, Schmidt SP, Zickler CF, Berner E, Damian LAK, McDonald Christian G, Workman H, Freeman M, Farley MD, Hicks TL: Team care of the patient with cleft lip and palate. *Curr Probl Pediatr Adolesc Health Care* 2008;38:138–158.
- Panetta NJ, Gupta DM, Slater BJ, Kwan MD, Liu KJ, Longaker MT: Tissue engineering in cleft palate and other congenital malformations. *Pediatr Res* 2008;63:545–551.
- Sitzman TJ, Girotto JA, Marcus JR: Current surgical practices in cleft care: unilateral cleft lip repair. *Plast Reconstr Surg* 2008;121:261e–270e.
- Wehby GL, Cassell CH: The impact of orofacial clefts on quality of life and healthcare use and costs. *Oral Dis* 2010;16:3–10.
- Chai Y, Maxson RE: Recent advances in craniofacial morphogenesis. *Dev Dyn* 2006;235:2353–2375.
- Iwata J, Parada C, Chai Y: The mechanism of TGF- β signaling during palate development. *Oral Dis* 2011;17:733–744.
- Dixon MJ, Marazita ML, Beaty TH, Murray JC: Cleft lip and palate: understanding genetic and environmental influences. *Nat Rev Genet* 2011;12:167–178.
- Sivertsen A, Wilcox AJ, Skjaerven R, Vindeas HA, Abyholm F, Harville E, Lie RT: Familial risk of oral clefts by morphological type and severity: population-based cohort study of first degree relatives. *BMJ* 2008;336:432–434.
- Jugessur A, Farlie PG, Kilpatrick N: The genetics of isolated orofacial clefts: from genotypes to subphenotypes. *Oral Dis* 2009;15:437–453.
- Cobourne MT: The complex genetics of cleft lip and palate. *Eur J Orthodont* 2004;26:7–16.
- Ardinger HH, Buetow KH, Bell GI, Bardach J, VanDemark DR, Murray JC: Association of genetic variation of the transforming growth factor- α gene with cleft lip and palate. *Am J Hum Genet* 1989;45:348–353.
- Jugessur A, Murray JC: Orofacial clefting: recent insights into a complex trait. *Curr Opin Genet Dev* 2005;15:270–278.
- Lidral AC, Moreno LM: Progress toward discerning the genetics of cleft lip. *Curr Opin Pediatr* 2005;17:731–739.
- Beaty TH, Murray JC, Marazita ML, Munger RG, Ruczinski I, Hetmanski JB, Liang KY, Wu T, Murray T, Fallin MD, Redett RA, Raymond G, Schwender H, Jin SC, Cooper ME, Dunnwald M, Mansilla MA, Leslie E, Bullard S, Lidral AC, Moreno LM, Menezes R, Vieira AR, Petrin A, Wilcox AJ, Lie RT, Jabs EW, Wu-Chou YH, Chen PK, Wang H, Ye X, Huang S, Yeow V, Chong SS, Jee SH, Shi B, Christensen K, Melbye M, Doheny KF, Pugh EW, Ling H, Castilla EE, Czeizel AE, Ma L, Field LL, Brody L, Pangilinan F, Mills JL, Molloy AM, Kirke PN, Scott JM, Arcos-Burgos M, Scott AF: A genome-wide association study of cleft lip with and without cleft palate identifies risk variants near MAFB and ABCA4. *Nat Genet* 2010;42:525–529.
- Lidral AC, Murray JC: Genetic approaches to identify disease genes for birth defects with cleft lip/palate as a model. *Birth Defects Res A Clin Mol Teratol* 2004;70:893–901.
- Marazita ML, Murray JC, Lidral AC, Arcos-Burgos M, Cooper ME, Goldstein T, Maher BS, Daack-Hirsch S, Schultz R, Mansilla MA, Field LL, Liu Y-e, Prescott N, Malcolm S, Winter R, Ray A, Moreno L, Valencia C, Neiswanger K, Wyszynski DF, Bailey-Wilson JE, Albacha-Hejazi H, Beaty TH, McIntosh I, Hetmanski JB, Tuncbilek G, Edwards M, Harkin L, Scott R, Roddick L: Meta-analysis of 13 genome scans reveals multiple cleft lip/palate genes with novel loci on 9q21 and 2q32–35. *Am J Hum Genet* 2004;75:161–173.
- Chiquet BT, Lidral AC, Stal S, Mulliken JB, Moreno LM, Arco-Burgos M, Valencia-Ramirez C, Blanton SH, Hecht JT: CRISPLD2: a novel NSCLP candidate gene. *Hum Mol Genet* 2007;16:2241–2248.
- Riley BM, Murray JC: Sequence evaluation of FGF and FGFR gene conserved non-coding elements in non-syndromic cleft lip and palate cases. *Am J Med Genet A* 2007;143A:3228–3234.
- Riley BM, Schultz RE, Cooper ME, Goldstein-McHenry T, Daack-Hirsch S, Lee KT, Dragan E, Vieira AR, Lidral AC, Marazita ML, Murray JC: A genome-wide linkage scan for cleft lip and cleft palate identifies a novel locus on 8p11–23. *Am J Med Genet A* 2007;143A:846–852.

- 21 Van den Boogaard MJH, Dorland M, Beemer FA, van Amstel HKP: MSX1 mutation is associated with orofacial clefting and tooth agenesis in humans. *Nat Genet* 2000;24:342–343.
- 22 Suzuki S, Marazita ML, Cooper ME, Miwa N, Hing A, Jugessur A, Natsume N, Shimozato K, Ohbayashi N, Suzuki Y, Niimi T, Minami K, Yamamoto M, Altannamar TJ, Erkhembataar T, Furukawa H, Daack-Hirsch S, L'Heureux J, Brandon CA, Weinberg SM, Neiswanger K, Deleyiannis FWB, de Salamanca JE, Vieira AR, Lidral AC, Martin JF, Murray JC: Mutations in BMP4 are associated with subepithelial, microform, and overt cleft lip. *Am J Hum Genet* 2009;84:406–411.
- 23 Murray JC: Gene/environment causes of cleft lip and/or palate. *Clin Genet* 2002; 61:248–256.
- 24 Wyszynski DF, Duffy DL, Beaty TH: Maternal cigarette smoking and oral clefts: a meta-analysis. *Cleft Palate Craniofac J* 1997;34:206–210.
- 25 Grewal J, Carmichael SL, Ma C, Lammer EJ, Shaw GM: Maternal periconceptional smoking and alcohol consumption and risk for select congenital anomalies. *Birth Defects Res A Clin Mol Teratol* 2008;82:519–526.
- 26 Lundqvist C, Zuurbier M, Leijis M, Johansson C, Ceccatelli S, Saunders M, Schoeters G, ten Tusscher G, Koppe JG: The effects of PCBs and dioxins on child health. *Acta Paediatr Suppl* 2006;95:55–64.
- 27 Castilla EE, Lopez-Camelo JS, Campaña H: Altitude as a risk factor for congenital anomalies. *Am J Med Genet* 1999;86:9–14.
- 28 Shahrukh Hashmi S, Gallaway MS, Waller DK, Langlois PH, Hecht JT: Maternal fever during early pregnancy and the risk of oral clefts. *Birth Defects Res A Clin Mol Teratol* 2010;88:186–194.
- 29 Beaty TH, Wang H, Hetmansk JB, Fan YT, Zeiger JS, Liang KY, Chiu YF, Vanderkolk CA, Seifert KC, Wulfsberg EA, Raymond G, Panny SR, McIntosh I: A case-control study of nonsyndromic oral clefts in Maryland. *Ann Epidemiol* 2001;11:434–442.
- 30 Honein MA, Rasmussen SA, Reefhuis J, Romitti PA, Lammer EJ, Sun L, Correa A: Maternal smoking and environmental tobacco smoke exposure and the risk of orofacial clefts. *Epidemiology* 2007;18:226–233.
- 31 Little J, Cardy A, Munger RG: Tobacco smoking and oral clefts: a meta-analysis. *Bull World Health Organ* 2004;82:213–218.
- 32 Shi M, Wehby GL, Murray JC: Review on genetic variants and maternal smoking in the etiology of oral clefts and other birth defects. *Birth Defects Res C Embryo Today* 2008;84:16–29.
- 33 Zeiger JS, Beaty TH, Liang K-Y: Oral clefts, maternal smoking, and TGFA: a meta-analysis of gene-environment interaction. *Cleft Palate Craniofac J* 2005;42:58–63.
- 34 Van Rooij IALM, Wegerif MJM, Roelofs HMJ, Peters WHM, Kuipers-Jagtman A-M, Zielhuis GA, Merkus HMWM, Steegers-Theunissen RgPM: Smoking, genetic polymorphisms in biotransformation enzymes, and nonsyndromic oral clefting: a gene-environment interaction. *Epidemiology* 2011;12:502–507.
- 35 DeRoo LA, Wilcox AJ, Drevon CA, Lie RT: First-trimester maternal alcohol consumption and the risk of infant oral clefts in Norway: a population-based case-control study. *Am J Epidemiol* 2008;168:638–646.
- 36 Boyles AL, DeRoo LA, Lie RT, Taylor JA, Jugessur A, Murray JC, Wilcox AJ: Maternal alcohol consumption, alcohol metabolism genes, and the risk of oral clefts: a population-based case-control study in Norway, 1996–2001. *Am J Epidemiol* 2010;172:924–931.
- 37 Jiang R, Bush JO, Lidral AC: Development of the upper lip: morphogenetic and molecular mechanisms. *Dev Dyn* 2006;235:1152–1166.
- 38 Ferguson MW: Palate development. *Development* 1988;103(suppl):41–60.
- 39 Chai Y, Jiang X, Ito Y, Bringas P, Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM: Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* 2000;127:1671–1679.
- 40 Ito Y, Yeo JY, Chytil A, Han J, Bringas P, Nakajima A, Shuler CF, Moses HL, Chai Y: Conditional inactivation of Tgfb2 in cranial neural crest causes cleft palate and calvaria defects. *Development* 2003;130:5269–5280.
- 41 Gato A, Martinez ML, Tudela C, Alonso I, Moro JA, Formoso MA, Ferguson MWJ, Martínez-Álvarez C: TGF- β_3 -induced chondroitin sulphate proteoglycan mediates palatal shelf adhesion. *Dev Biol* 2002;250:393–405.
- 42 Cuervo R, Covarrubias L: Death is the major fate of medial edge epithelial cells and the cause of basal lamina degradation during palatogenesis. *Development* 2004;131:15–24.
- 43 Jin J-Z, Ding J: Analysis of cell migration, transdifferentiation and apoptosis during mouse secondary palate fusion. *Development* 2006;133:3341–3347.
- 44 Vaziri Sani F, Hallberg K, Harfe BD, McMahon AP, Linde A, Gritli-Linde A: Fate-mapping of the epithelial seam during palatal fusion rules out epithelial-mesenchymal transformation. *Dev Biol* 2005;285:490–495.
- 45 Xu X, Han J, Ito Y, Bringas P Jr, Urata MM, Chai Y: Cell autonomous requirement for Tgfb2 in the disappearance of medial edge epithelium during palatal fusion. *Dev Biol* 2006;297:238–248.
- 46 Meng L, Bian Z, Torensmar R, Von den Hoff JW: Biological mechanisms in palatogenesis and cleft palate. *J Dent Res* 2009;88:22–33.
- 47 Derynck R, Zhang YE: Smad-dependent and Smad-independent pathways in TGF- β family signalling. *Nature* 2003;425:577–584.
- 48 Shi Y, Massagué J: Mechanisms of TGF- β signaling from cell membrane to the nucleus. *Cell* 2003;113:685–700.
- 49 Kishigami S, Mishina Y: BMP signaling and early embryonic patterning. *Cytokine Growth Factor Rev* 2005;16: 265–278.
- 50 Nie X, Luukko K, Kettunen P: BMP signalling in craniofacial development. *Int J Dev Biol* 2006;50:511–521.
- 51 Sieber C, Kopf J, Hiepen C, Knaus P: Recent advances in BMP receptor signalling. *Cytokine Growth Factor Rev* 2009; 20:343–355.
- 52 Nohe A, Keating E, Knaus P, Petersen NO: Signal transduction of bone morphogenetic protein receptors. *Cell Signal* 2004;16:291–299.
- 53 Alappat S, Zhang ZY, Chen YP: Msx homeobox gene family and craniofacial development. *Cell Res* 2003;13:429–442.
- 54 Gazzero E, Canalis E: Bone morphogenetic proteins and their antagonists. *Rev Endocr Metab Disord* 2006;7:51–65.
- 55 Hartung A, Bitton-Worms K, Rechtman MM, Wenzel V, Boergermann JH, Hassel S, Henis YI, Knaus P: Different routes of bone morphogenetic protein (BMP) receptor endocytosis influence BMP signaling. *Mol Cell Biol* 2006;26:7791–7805.

- 56 Halbrooks P, Ding R, Wozney J, Bain G: Role of RGM coreceptors in bone morphogenetic protein signaling. *J Mol Signal* 2007;2:4.
- 57 Onichtchouk D, Chen Y-G, Dosch R, Gawantka V, Delius H, Massague J, Niehrs C: Silencing of TGF- β signalling by the pseudoreceptor BAMBI. *Nature* 1999;401:480–485.
- 58 Sammar M, Stricker S, Schwabe GC, Sieber C, Hartung A, Hanke M, Oishi I, Pohl J, Minami Y, Sebald W, Mundlos S, Knaus P: Modulation of GDF5/BRI-b signalling through interaction with the tyrosine kinase receptor Ror2. *Genes Cells* 2004;9:1227–1238.
- 59 Wu KM, Huang CJ, Hwang SP, Chang YS: Molecular cloning, expression and characterization of the zebrafish *bram1* gene, a BMP receptor-associated molecule. *J Biomed Sci* 2006;13:345–355.
- 60 Yamaguchi K, Nagai S-I, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H, Matsumoto K: XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. *EMBO J* 1999;18:179–187.
- 61 Wan M, Cao X: BMP signaling in skeletal development. *Biochem Biophys Res Commun* 2005;328:651–657.
- 62 Francis-West PH, Tatla T, Brickell PM: Expression patterns of the bone morphogenetic protein genes *Bmp-4* and *Bmp-2* in the developing chick face suggest a role in outgrowth of the primordia. *Dev Dyn* 1994;201:168–178.
- 63 Barlow AJ, Francis-West PH: Ectopic application of recombinant BMP-2 and BMP-4 can change patterning of developing chick facial primordia. *Development* 1997;124:391–398.
- 64 Brugger SM, Merrill AE, Torres-Vazquez J, Wu N, Ting M-C, Cho JY-M, Dobias SL, Yi SE, Lyons K, Bell JR, Arora K, Warrior R, Maxson R: A phylogenetically conserved *cis*-regulatory module in the *Msx2* promoter is sufficient for BMP-dependent transcription in murine and *Drosophila* embryos. *Development* 2004;131:5153–5165.
- 65 Gong SG, Guo C: *Bmp4* gene is expressed at the putative site of fusion in the midfacial region. *Differentiation* 2003;71:228–236.
- 66 Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen Y: Rescue of cleft palate in *Msx1*-deficient mice by transgenic *Bmp4* reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. *Development* 2002;129:4135–4146.
- 67 Han J, Mayo J, Xu X, Li J, Bringas P, Maas RL, Rubenstein JLR, Chai Y: Indirect modulation of Shh signaling by *Dlx5* affects the oral-nasal patterning of palate and rescues cleft palate in *Msx1*-null mice. *Development* 2009;136:4225–4233.
- 68 Ashique AM, Fu K, Richman JM: Endogenous bone morphogenetic proteins regulate outgrowth and epithelial survival during avian lip fusion. *Development* 2002;129:4647–4660.
- 69 Wu P, Jiang T-X, Suksaweang S, Widelitz RB, Chuong C-M: Molecular shaping of the beak. *Science* 2004;305:1465–1466.
- 70 Helms JA, Cordero D, Tapadia MD: New insights into craniofacial morphogenesis. *Development* 2005;132:851–861.
- 71 Mishina Y, Hanks MC, Miura S, Tallquist MD, Behringer RR: Generation of *Bmpr/Alk3* conditional knockout mice. *Genesis* 2002;32:69–72.
- 72 Mishina Y, Suzuki A, Ueno N, Behringer RR: *Bmpr* encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. *Genes Dev* 1995;9:3027–3037.
- 73 Winnier G, Blessing M, Labosky PA, Hogan BL: Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev* 1995;9:2105–2116.
- 74 Liu W, Sun X, Braut A, Mishina Y, Behringer RR, Mina M, Martin JF: Distinct functions for *Bmp* signaling in lip and palate fusion in mice. *Development* 2005;132:1453–1461.
- 75 Baek J-A, Lan Y, Liu H, Maltby KM, Mishina Y, Jiang R: *Bmpr1a* signaling plays critical roles in palatal shelf growth and palatal bone formation. *Dev Biol* 2011;350:520–531.
- 76 Dudas M, Sridurongrit S, Nagy A, Okazaki K, Kaartinen V: Craniofacial defects in mice lacking BMP type I receptor *Alk2* in neural crest cells. *Mech Dev* 2004;121:173–182.
- 77 Sirard C, de la Pompa JL, Elia A, Itie A, Mirtsos C, Cheung A, Hahn S, Wakeham A, Schwartz L, Kern SE, Rossant J, Mak TW: The tumor suppressor gene *Smad4/Dpc4* is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev* 1998;12:107–119.
- 78 Yang X, Li C, Xu X, Deng C: The tumor suppressor *SMAD4/DPC4* is essential for epiblast proliferation and mesoderm induction in mice. *Proc Natl Acad Sci USA* 1998;95:3667–3672.
- 79 Xu X, Han J, Ito Y, Bringas P Jr, Deng C, Chai Y: Ectodermal *Smad4* and p38 MAPK Are functionally redundant in mediating TGF- β /BMP signaling during tooth and palate development. *Dev Cell* 2008;15:322–329.
- 80 Ko SO, Chung IH, Xu X, Oka S, Zhao H, Cho ES, Deng C, Chai Y: *Smad4* is required to regulate the fate of cranial neural crest cells. *Dev Biol* 2007;312:435–447.
- 81 Satokata I, Maas RL: *Msx1*-deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nat Genet* 1994;6:348–356.
- 82 Ishii M, Han J, Yen H-Y, Sucov HM, Chai Y, Maxson RE: Combined deficiencies of *Msx1* and *Msx2* cause impaired patterning and survival of the cranial neural crest. *Development* 2005;132:4937–4950.
- 83 Levi G, Mantero S, Barbieri O, Cantatore D, Paleari L, Beverdam A, Genova F, Robert B, Merlo GR: *Msx1* and *Dlx5* act independently in development of craniofacial skeleton, but converge on the regulation of *Bmp* signaling in palate formation. *Mech Dev* 2006;123:3–16.
- 84 He F, Xiong W, Wang Y, Matsui M, Yu X, Chai Y, Klingensmith J, Chen Y: Modulation of BMP signaling by *Noggin* is required for the maintenance of palatal epithelial integrity during palatogenesis. *Dev Biol* 2010;347:109–121.
- 85 Wyatt AW, Osborne RJ, Stewart H, Ragge NK: Bone morphogenetic protein 7 (BMP7) mutations are associated with variable ocular, brain, ear, palate, and skeletal anomalies. *Hum Mutat* 2010;31:781–787.
- 86 Lidral AC, Murray JC, Buetow KH, Basart AM, Scheerer H, Shiang R, Naval A, Layda E, Magee K, Magee W: Studies of the candidate genes *TGFB2*, *MSX1*, *TGFA*, and *TGFB3* in the etiology of cleft lip and palate in the Philippines. *Cleft Palate Craniofac J* 1997;34:1–6.

- 87 Jezewski PA, Vieira AR, Nishimura C, Ludwig B, Johnson M, O'Brien SE, Daack-Hirsch S, Schultz RE, Weber A, Nepomucena B, Romitti PA, Christensen K, Orioli IM, Castilla EE, Machida J, Natsume N, Murray JC: Complete sequencing shows a role for MSX1 in nonsyndromic cleft lip and palate. *J Med Genet* 2003;40:399–407.
- 88 Suzuki Y, Jezewski PA, Machida J, Watanabe Y, Shi M, Cooper ME, Viet LT, Tin NTD, Hai H, Natsume N, Shimoizato K, Marazita ML, Murray JC: In a Vietnamese population, MSX1 variants contribute to cleft lip and palate. *Genet Med* 2004;6:117–125.
- 89 Vieira AR, Castillo Taucher S, Aravena T, Astete C, Sanz P, Tastets ME, Monasterio L, Murray JC: Mutational analysis of the muscle segment homeobox gene 1 (MSX1) in Chilean patients with cleft lip/palate. *Rev Med Chil* 2005;132:816–822.
- 90 Singh VP, Ramu D: Association of MSX1 799 G>T variant with nonsyndromic cleft lip/palate in South Indian adolescent patients. *Int J Paediatr Dent* 2011 Oct 4 doi: [10.1111/j.1365-263X.2011.01184.x](https://doi.org/10.1111/j.1365-263X.2011.01184.x) (E-pub ahead of print).
- 91 Salahshourifar I, Halim AS, Wan Sulaiman WA, Zilfalil BA: Contribution of MSX1 variants to the risk of nonsyndromic cleft lip and palate in a Malay population. *J Hum Genet* 2011;56:755–758.
- 92 Huang Y-Q, Ma J, Ma M, Deng Y, Li Y-D, Ren H-W, Zhao G-Z, Guo S-S, Wang Y-Y, Zhang G-X, Shi B: Association between MSX1 variants and oral clefts in Han Chinese in Western China. *DNA Cell Biol* 2011;30:1057–1061.
- 93 Jagomägi T, Nikopensius T, Krjutškov K, Tammekivi V, Viltrop T, Saag M, Metspalu A: MTHFR and MSX1 contribute to the risk of nonsyndromic cleft lip/palate. *Eur J Oral Sci* 2010;118:213–220.
- 94 Otero L, Gutiérrez S, Chaves M, Vargas C, Bermudez L: Association of MSX1 with nonsyndromic cleft lip and palate in a Colombian population. *Cleft Palate Craniofac J* 2007;44:653–656.

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Development of the Lip and Palate: FGF Signalling

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Abstract

The fibroblast growth factor (FGF) signalling pathway is critically involved in several aspects of craniofacial development, including formation of the lip and the palate. FGF receptors are activated by extracellular FGF ligands in order to regulate cellular processes such as migration and morphogenesis through instruction of specific target gene expression. A key factor in the development of orofacial structures is the interaction between mesodermal- and neural crest-derived mesenchyme and ecto- and endodermal-derived epithelium. FGF signalling occurs in both cell types and promotes epithelial-mesenchymal communication through region-specific expression of receptor subtypes. Many FGF ligands and receptors are expressed at specific stages and at precise locations during normal palatogenesis and an absolute requirement of some has been demonstrated by their (conditional) inactivation resulting in a cleft palate phenotype. Other important signalling pathways involving SHH and SPRY are intricately involved in the interpretation of FGF signalling. As a cause of human pathology, functionally validated FGF pathway gene mutations have been exclusively associated with syndromic forms of cleft lip and palate. Most commonly, this includes patients with mutations in *FGFR1* and *FGFR2* (Kallmann, Pfeiffer, Apert and Crouzon syndromes) where cleft palate is part of a broad craniofacial phenotype, including craniosynostosis. Similarly, *FGF8* mutations have been found in patients with Kallmann-like idiopathic hypogonadotropic hypogonadism, some also with cleft lip and palate. In this chapter, we

will provide an overview of the relevant FGF ligands and receptors important for lip and palate morphogenesis, correlating their expression patterns with the effects of their perturbation that lead to a clefting pathogenesis.

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One of the most important, and therefore one of the most studied signalling pathways required for normal embryonic development is that involving the fibroblast growth factor (FGF) family and its receptors. When extracellular FGF ligands bind to FGF receptors at the membrane of target cells, intracellular FGF signalling can mediate a wide range of different biological processes. During development it plays a crucial role by regulating proliferation, differentiation and cell mobility. In addition, many other classical signalling pathways involving SHH, BMP and WNT proteins are closely allied to FGF signalling during embryonic development, and have been shown to cross-regulate cellular events required for normal craniofacial morphogenesis. The ubiquitous spatio-temporal expression patterns of many FGF ligands and receptors implicate an essential role for this signalling pathway, from gastrulation to adult homeostasis, but in this chapter we will focus on its role during lip and palate development.

FGF signalling is critically involved in several aspects of craniofacial development, and is particularly known for its role in regulating neural

crest induction and migration, skeletogenesis and epithelial-mesenchymal interactions. Our current understanding of individual FGF ligand and receptor function has mostly been acquired through the study of knockout transgenics, where specific FGF pathway genes targeted for mutagenesis result in an animal lacking the corresponding functional protein. In the past decade, FGF signalling pathway receptors, more commonly than their ligands, have been shown to contribute significantly to the pathogenesis of several well-characterised syndromic birth defects, which frequently include cleft lip and/or palate (CL/P) as a characteristic feature. In contrast, mutations in FGF receptors or ligand are rarely causative for non-syndromic CL/P.

Basic Mechanisms of FGF Signalling

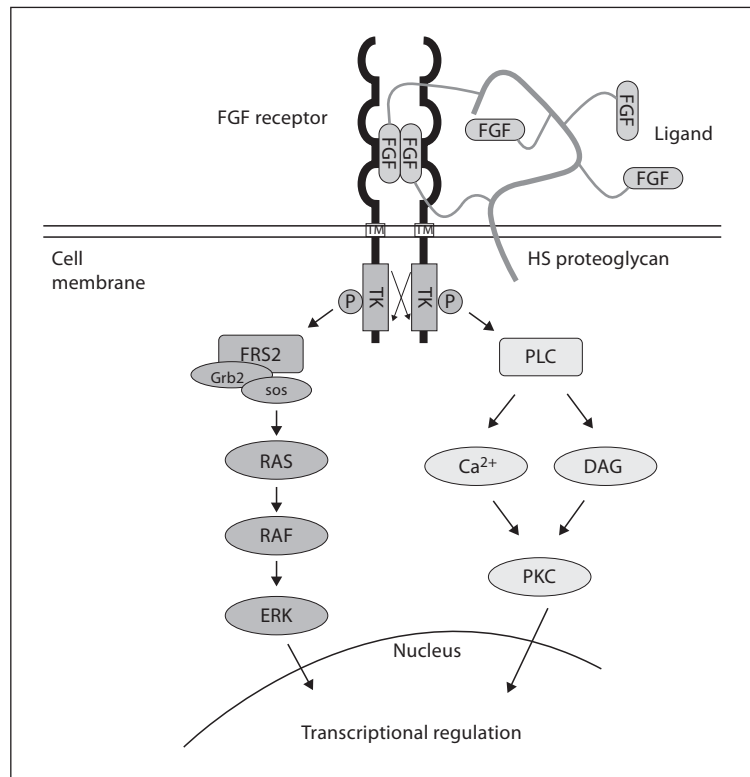
The general mode of action of FGF ligands and their receptors is conserved throughout metazoan evolution. Studies in *Drosophila melanogaster* and *Caenorhabditis elegans* have shown that FGF receptors (encoded by homologous genes *heartless/breathless* in the fly and *egl-15* in the worm) are activated by extracellular FGF ligands and modulated by heparan sulfate proteoglycans (HSPG) in order to regulate cellular processes such as migration and morphogenesis. Regulation is complex, such that tissue and cell-specific expression of these genes are often controlled through alternative splicing [1]. As a result of various genomic duplications during vertebrate evolution, a total of 22 FGF ligands and 4 FGF receptors can now be identified in humans and mice. Most FGF ligands are secreted and function extracellularly, with the exception of FGFs 11–14, which is a subfamily of proteins that have intracellular, receptor independent functions [2]. The other 18 FGF ligands have unique binding affinities for individual FGF receptor isoforms, illustrating their specific function and spatio-temporal expression.

FGF receptors are transmembrane proteins with an extracellular domain that consists of

three immunoglobulin (Ig)-like structures. Crystal structures and biochemical studies have shown that the ligand-binding domain is likely to lie between Ig loops II and III. The intracellular domain contains a tyrosine kinase unit that is involved in the phosphorylation of tyrosine residues of receptor complexes upon ligand binding and dimerization. Alternative splicing of the genes encoding FGF receptors 1, 2 and 3 results in two isoforms differing in only their third immunoglobulin (Ig) loop (IIIb and IIIc). The IIIb variant is mainly expressed in epithelium, whereas IIIc is expressed in mesenchyme. The unique binding properties of both isoform types make it possible for specific FGF ligands to signal to specific developing regions, especially where epithelial-mesenchymal interactions occur [3]. An essential requirement for FGF signalling is the presence of HSPG that bind both the ligand and the receptor. The presence or absence of HSPG in different tissues may affect the activity of FGFs and play a role in the formation of ternary extracellular signalling complexes [4].

The main downstream, intracellular target of the FGF-FGFR-HSPG complex is fibroblast growth factor receptor substrate 2 (FRS2). After receptor complex activation through autophosphorylation, a signalling cascade is formed through FRS2 and the canonical RAS-ERK pathway, eventually leading to appropriate regulation of gene expression in the cell nucleus. FRS2 is located on the cell membrane and converges FGF signals from receptor to intracellular kinases. In addition to canonical FGF signalling, FGF receptor complexes are also capable of signalling via phospholipase C (PLC) to increased levels of either intracellular Ca^{2+} or diacylglycerol (DAG) causing activation of protein kinase C (PKC) and regulation of gene expression (fig. 1). Furthermore, FGF signalling in mammalian development is closely integrated with other pathways such as BMP, SHH, TGF β , SOX, MSX, DLX, EGF and WNT, which are all known to be required for correct craniofacial development [5].

Fig. 1. Basic FGF signalling pathway. FGF ligand molecules bound to the heparan sulfate (HS) proteoglycans in the extracellular matrix are presented to membrane-bound FGF receptors (TM). Upon binding, receptors dimerise to cross-modulate phosphorylation (P) at the intracellular tyrosine kinase (TK) domains. Activated receptors signal downstream through the canonical FRS/RAS/ERK pathway, or the non-canonical Ca^{2+} /DAG pathway via PLC and PKC, resulting in transcriptional regulation in the nucleus.



Role of FGF Signalling in the Embryonic Development of the Lip and Palate

Formation of the vertebrate face is a complex process involving outgrowth and differentiation of the facial primordia, which is orchestrated by various inductive signals. Craniofacial defects are an inevitable outcome of a failure in correct FGF signalling, as FGF ligands and receptors are involved in diverse stages of development. The earliest event is cranial neural crest (CNC) cell induction, which involves cells originating from the ectoderm overlying the dorsal ridges of the neural tube [6]. Subsequently, these cells migrate into the pharyngeal arches and populate the frontonasal, maxillary and mandibular primordia. These tissues result from FGF-directed condensation of

the CNC and are responsible for the development of the majority of facial structures including the cranial cartilage and skeleton, which also contribute to the upper and lower jaw, lip and palate. A key factor in this morphogenic process is the interaction between mesoderm- and neural crest-derived mesenchyme and ecto- and endoderm-derived epithelium. FGF signalling occurs in both cell types and promotes reciprocal epithelial-mesenchymal communication through region-specific expression of FGF receptor isoforms.

In humans, loss-of-function mutations in FGFR 1, 2 and 3 are commonly associated with craniosynostosis and other facio-skeletal malformations, including cleft palate (CP) [7]. Gain-of-function mutations in *FGF10* are mainly associated with the absence of the salivary glands [8],

Table 1. Targeted mutation of FGF(R) genes in mouse transgenic models

Gene	Phenotype	MGI no. ¹	Ref.
<i>Fgfr1</i>	Conditional (Wnt-1-Cre) FGFR1b loss of function animals have cleft palate	95522	17
<i>Fgfr2</i>	Loss of function of the FGFR2b isoform results in cleft palate Gain of function C342Y mutation in FGFR2c results in cleft palate in homozygous animals	95523	12, 18 19
<i>Fgf8</i>	Pharyngeal arch hypoplasia, cleft palate	99604	20, 21
<i>Fgf10</i>	Multi-organ dysgenesis, cleft palate	1099809	11, 18
<i>Fgf18</i>	Delayed ossification, cleft palate	1277980	14
<i>Cfr</i>	Growth retardation, cleft palate	104967	22

¹ Mouse Genome Informatics database at the Jackson laboratory: <http://www.informatics.jax.org/>

while nonsense mutations in FGF8 are linked to the occurrence of CP in patients with isolated hypogonadotropic hypogonadism [9]. In mouse, some FGF(R)s are highly expressed in the tissues that form the upper lip and palate. As the primitive face is taking shape, *Fgf8* is expressed quite specifically in the frontonasal prominence, in a region where the nasal pits invaginate and at the precise point at which the medial and lateral nasal prominences fuse, forming the lip and primary palate [10]. Within the secondary palate, *Fgfr1*, *Fgfr2*, *Fgfr3*, *Fgf10* and *Fgf18* are expressed during various developmental stages, notably the palatal epithelium at the time of outgrowth and both the proliferating palatal mesenchyme and the medial edge epithelial (MEE) cells during the process of palatal fusion [11–14]. As such, one would expect these members of the FGF signalling pathway to provide strong candidate genes for a role in the formation of the lip and the palate. Perhaps surprisingly, in mice deficient for various FGF ligands, defects of the lip are rarely seen although CP is a common feature. Similar findings have also been observed in transgenic studies with other key signalling molecules of the BMP, SHH, MSX, TGF β

and EGF signalling pathways [15]. This suggests that in the mouse, morphogenesis of the lip is more resistant to genetic alteration than that of the palate. In contrast, mouse models deficient for genes causing CP only are often associated with CL/P in the human condition [16]. One possible explanation is the rodent's craniofacial anatomy, which differs slightly from humans in terms of the abundant midfacial tissue, rather than a redundant or non-functional role. An overview of FGF and FGFR function based on transgenic mouse models is presented in table 1.

FGF Receptors

Fgfr1 is expressed in the developing mouse pharyngeal arches and persists until later stages, specifically the isoform *Fgfr1b*, which is found in the epithelial layer of the palatal shelves. The corresponding targeted mouse mutant causing loss of FGFR1b function results in CP [17], confirming its essential role in this tissue. Similarly, the homozygous *Fgfr2b* loss of function mutant also exhibits CP [12]. In the latter case, the potential mechanism was shown to involve decreased FGF10-FGFR2b signalling and subsequent misregulation of SHH

expression in the epithelial cells of the palatal shelves [18]. Meanwhile, a gain-of-function mouse mutant displaying a phenotype resembling human Crouzon syndrome was also found to have CP [19]. This particular mutant affected only the FGFR2c isoform, which is expressed in palatal mesenchyme. Although at the biochemical level the mutation appears to be activating, evidence suggests that the net result could be a loss of FGF signalling [23]. The underlying mechanisms involved in the epithelial-mesenchymal signalling processes are still to be uncovered, although it is clearly sensitive to the level of FGF signalling during palate development.

In the human palate, *FGFR1* and *FGFR2* mRNA transcripts and their corresponding proteins have been detected specifically in the MEE cells of fusing palatal shelves [24]. This suggests an important role for these FGF receptors in correct human palate formation. Certainly, in the mouse palate, strong expression of FGFR1 and 2 proteins were detected in the epithelium peri-fusion, as well as in the post-fusion pre-osteogenic mesenchyme [25]. Although the FGFR3 or 4 receptors have not been associated with lip and palate development, another, cysteine-rich FGF receptor homologue (CFR), was found to develop a cleft palate in a recently described mouse mutant [22]. This distant homologue of the four main FGF receptors appears to interact with FGF18 and presents a novel regulatory mechanism for FGF signalling in the palate.

FGF Ligands

Many of the FGF ligands have been extensively studied for their craniofacial expression in humans and mouse, with specific expression patterns during lip and palate development reported. *FGF2* has been shown to be expressed in MEE cells of the human palate [24], but also in the mesenchyme of mouse palatal shelves [26]. *Fgf3* is expressed in the epithelium of developing palatal shelves [26], while *FGF4* is expressed in the MEE cells of the developing human palate.

Perhaps surprisingly, none of these showed phenotypes affecting the lip or palate when the respective genes were knocked out. *Fgf6* is also expressed in the epithelial cells of the developing mouse palate [26] while *Fgf7* is expressed in mouse palatal mesenchyme [18], as well as in the MEE cells in the human palate [24]. Again, loss-of-function for these genes does not appear to result in a lip or palate phenotype. A notable exception is *Fgf8*, which is expressed in the ectoderm during pharyngeal arch development [20, 27], as well as in the mesenchyme of developing palatal shelves [28, 26]. FGF8-deficient mice show defects in CNC-derived tissues, including CP [21]. This data was supported by a study in which *Fgf8* haploinsufficiency in zebrafish also caused craniofacial malformations [29]. Tissue-specific inactivation has shown FGF8 to be essential for the formation of midfacial mesenchyme, as mutant mice show pharyngeal arch hypoplasia as well as CP [20]. Biochemical studies have shown FGF8 to be a ligand for mesenchymal FGFR3c isoforms [30]. Therefore, it is likely that FGF8 performs its main function as a ligand in the epithelium of the developing palate through reciprocal paracrine signalling, but it is possible that FGF8 is able to function independent of receptor activation in the mesenchyme. The latter is supported by data showing *Fgf8* to be expressed as eight different splice variants with distinct functions [31]. Meanwhile, FGF10 is probably the FGF ligand that is most extensively studied in the development of the palate. Its expression domain has been shown to extend throughout the palatal mesenchyme from E11.5–13.5, which spans the period of palatal shelf proliferation and elevation [11]. The cleft phenotype found in *Fgf10* knockout mice is very similar to that of *Fgfr2b*-deficient mice, where a failure of shelf growth through decreased levels of proliferation has been shown to be resulting from a lack of SHH signalling, while premature fusion of palatal epithelium with the epithelial cells of the tongue might be another cause of the cleft palate phenotype [18]. The

Table 2. *FGF* and *FGFR* genes that cause CL/P in human mendelian disorders

Gene	CL/P-associated human disorder	OMIM no. ¹	Ref.
<i>FGFR1</i>	Kallmann syndrome (KAL2): hypogonadism, anosmia; cleft lip and/or palate Pfeiffer: craniosynostosis, acrocephalopolysyndactyly, craniofacial defects; high-arched or cleft palate	136350	38, 39
<i>FGFR2</i>	Apert: craniosynostosis, syndactyly, craniofacial defects; high-arched or cleft palate Crouzon: craniosynostosis, hypertelorism, craniofacial defects; high-arched or cleft palate	176943	40 41
<i>FGF8</i>	Isolated hypogonadotropic hypogonadism; cleft lip and palate	600483	9

¹ Online Mendelian Inheritance in Man database at NCBI: <http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM/>

epithelial-mesenchymal interactions between FGF10 and FGFR2b have been described as essential for correct growth and differentiation at many other different stages of development, including the limb [32] and organogenesis [33]. Finally, *Fgf18* is expressed in the mesenchyme of palatal shelves before fusion and loss of function mutants display cleft palate [14]. While FGF18 is more commonly associated with endochondral chondro- and osteogenesis [34], it has been associated with induction of RUNX1 in the MEE cells of palatal shelves during fusion, and *Runx1*-deficient mice show anterior clefts of the palate [35]. It is unclear however how FGF18 signals from the mesenchyme to the epithelium in the palate, as it is shown to be a ligand for the mesenchymal FGFR1-3c isoforms.

It is now clear that reciprocal FGF signalling between the adjacent palatal epithelium and mesenchyme is an essential process during normal palatogenesis. The correct dosage of FGF signalling is vital for a normal progression of the three stages of palate formation: growth, elevation and fusion. Disturbances in any of these processes can cause cleft palate and FGF signalling has been involved in all of them. Another indication that precise levels of FGF signalling are essential is the fact

that mice deficient for the FGF antagonist SPRY2 show cleft palate with increased levels of FGF signalling and subsequent proliferation of early palatal shelf outgrowth [36, 37].

FGF Pathway Mutations and the Pathogenesis of Human CLP

In humans, several genes encoding FGFs and FGFRs have important roles in several aspects of craniofacial development including the lip and palate, which are best highlighted by the syndromic birth defects resulting from disorders associated with FGF pathway gene mutations (table 2). CL/P is seen as a common feature in many of the FGF-related craniosynostosis syndromes, being present in 25–30% of patients with the autosomal dominant form of Kallmann syndrome (KAL2) caused by *FGFR1* mutations [38]. To date, at least 14 different mutations in *FGFR1* have been described in KAL2. One of these was a mutation in the FGFR1c isoform found in a Kallmann syndrome patient with CP, suggesting a role for this particular FGFR in the development of the secondary palate [42]. This was corroborated by a study showing that mice homozygous

for a hypomorphic allele of *Fgfr1* have craniofacial defects, including CP [17]. The X-linked form of Kallmann syndrome (KAL1) is characterised by a high-arched palate, and mutations in *KAL1* (encoding the Anosmin-1 protein) have also been identified [39]. It is suggested that Anosmin-1 is involved in FGF signalling as it has been shown to bind to the extracellular domain of FGFR1 [38].

Germline mutations in the *FGFR2* gene have been shown to cause dominant mendelian disorders associated with craniosynostosis and additional craniofacial features, including CP. CP or bifid uvula was reported in 75% of patients with Apert syndrome, with a Byzantine arch-shaped palate in almost all [43]. In Crouzon syndrome, CP is much less frequent, which is probably a reflection of the fact that Apert mutations affect both FGFR2 isoforms, while Crouzon mutations typically affect only the mesenchymal FGFR2c isoform. Mouse studies have shown that the epithelial FGFR2b isoform is essential for correct palate malformation [18]. Similarly, patients with Pfeiffer syndrome, due to dominant mutations in *FGFR1*, have high levels of high-arched palate (87%) with occasional CP [44]. The mutation responsible for Pfeiffer syndrome affects a synonymous codon (P252 for Pfeiffer and P253 for Apert) in the homologous receptors.

In spite of a body of evidence for the expression of several FGF and FGFR genes during orofacial development and the requirement of FGF signalling during palatogenesis in animal models, not all of those genes give rise to a clefting phenotype in loss-of-function models (see above). It may therefore be no surprise that few FGF pathway genes have been found to be responsible for human non-syndromic CL/P. At the same time, mutations causing CP have been described in many other genes involved in development (*TBX22* [45], *MSX1* [46], *IRF6* [47]), some of which are linked to, or directly downstream of FGF signalling. For example, *Tbx22* expression has been shown to be regulated by FGF8 during the development of the palate in the chicken and the mouse [28].

A more systematic analysis of FGF(R) family member gene sequences was conducted, analysing 12 FGF(R) genes in 184 patients with non-syndromic CL/P [48]. This study identified a low frequency of putative mutations primarily in the receptors *FGFR1* (p.M369I, p.E467K) and *FGFR2* (p.R84S, p.D138N), but also in the ligand *FGF8* (p.D73H). This work potentially highlights an important role of receptors during palatogenesis, while suggesting that ligands play a rare or minor role in non-syndromic patients. Nonsense mutations were also found in Kallmann syndrome patients with CLP, both in this study in *FGFR1* (p.R609X) and in a separate study in *FGF8* (p.R127X) [9], highlighting the potential overlap between non-syndromic CL/P and Kallmann syndrome. In addition to sequencing, association tests were also performed with FGF-specific single nucleotide polymorphisms. Here, missense mutations were over-represented in patients compared to controls, suggesting a possible role for *FGF3*, *FGF7*, *FGF10*, *FGF18* and *FGFR1* in the CL/P phenotype. Although it is more difficult to pin down the exact mechanism for variants of low penetrance, presumably interacting with other genetic and environmental risk factors, their frequency in the population might account for a much higher proportion of CL/P than the more severe, but rare variants [49].

Conclusions and Future Directions

We can conclude that FGF signalling is essential for normal development of the lip and especially the palate. Studies in animal models have revealed that the specific ligands FGF8, FGF10, FGF18 and the receptors FGFR1 and FGFR2 are the most important for normal palatogenesis. However, the precise processes that FGF signalling affects or through which target genes this is regulated, still remains to be fully appreciated.

In terms of a genetic explanation to human orofacial pathology, mutations have been

described in human FGF genes that are primarily associated with syndromic forms of CL/P. These include patients with mutations in *FGFR1* and *FGFR2*, where Kallmann, Pfeiffer, Apert and Crouzon syndromes can include CP as part of a broader craniofacial phenotype, including craniosynostosis, midfacial hypoplasia and hypertelorism. It could be argued that the palate defects may be a secondary to extensive dysmorphology of the head, but it is likely also to be an indication of the sensitivity of the process of palatogenesis for misregulation of FGF signalling. The rarity of FGF mutations that have been identified in non-syndromic CL/P could also be a reflection of the fact that FGF signalling is simply too important and too ubiquitous to be disrupted by a loss of

function mutation. This is illustrated by the fact that some FGF(R) transgenic models only reveal a clefting phenotype when conditional inactivation is employed.

Future studies will focus on genomic association studies, perhaps alongside exome sequencing, to identify multiple polymorphic mutations in FGF and/or other genes expressed in the lip and palate that in combination are sufficient to disturb the normal growth and fusion process of the midface. At the same time, detailed studies of the intricate mechanisms of FGF signalling using conditional knockout technology in mammalian model systems will lead to novel protein partners and downstream candidate genes.

References

- Huang P, Stern MJ: FGF signaling in flies and worms: more and more relevant to vertebrate biology. *Cytokine Growth Factor Rev* 2005;16:151–158.
- Itoh N, Ornitz DM: Evolution of the Fgf and Fgfr gene families. *Trends Genet* 2004;20:563–569.
- Eswarakumar VP, Lax I, Schlessinger J: Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev* 2005;16:139–149.
- Harmer NJ: Insights into the role of heparan sulphate in fibroblast growth factor signalling. *Biochem Soc Trans* 2006;34:442–445.
- Nie X, Luukko K, Kettunen P: FGF signalling in craniofacial development and developmental disorders. *Oral Dis* 2006;12:102–111.
- Knecht AK, Bronner-Fraser M: Induction of the neural crest: a multigene process. *Nat Rev Genet* 2002;3:453–461.
- Ibrahimi OA, Zhang F, Eliseenkova AV, Linhardt RJ, Mohammadi M: Proline to arginine mutations in FGF receptors 1 and 3 result in Pfeiffer and Muenke craniosynostosis syndromes through enhancement of FGF binding affinity. *Hum Mol Genet* 2004;13:69–78.
- Entesarian M, Matsson H, Klar J, Bergendal B, Olson L, Arakaki R, Hayashi Y, Ohuchi H, Falahat B, Bolstad AI, Jonsson R, Wahren-Herlenius M, Dahl N: Mutations in the gene encoding fibroblast growth factor 10 are associated with aplasia of lacrimal and salivary glands. *Nat Genet* 2005;37:125–127.
- Trarbach EB, Abreu AP, Silveira LF, Garmes HM, Baptista MT, Teles MG, Costa EM, Mohammadi M, Pitteloud N, Mendonca BB, Latronico AC: Nonsense mutations in FGF8 gene causing different degrees of human gonadotropin-releasing deficiency. *J Clin Endocrinol Metab* 2010;95:3491–3496.
- Bachler M, Neubuser A: Expression of members of the Fgf family and their receptors during midfacial development. *Mech Dev* 2001;100:313–316.
- Alappat SR, Zhang Z, Suzuki K, Zhang X, Liu H, Jiang R, Yamada G, Chen Y: The cellular and molecular etiology of the cleft secondary palate in Fgf10 mutant mice. *Dev Biol* 2005;277:102–113.
- De Moerloose L, Spencer-Dene B, Revest J, Hajihosseini M, Rosewell I, Dickson C: An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. *Development* 2000;127:483–492.
- Rice R, Spencer-Dene B, Connor EC, Gritli-Linde A, McMahon AP, Dickson C, Thesleff I, Rice DP: Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. *J Clin Invest* 2004;113:1692–1700.
- Liu Z, Xu J, Colvin JS, Ornitz DM: Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. *Genes Dev* 2002;16:859–869.
- Wilkie AO, Morriss-Kay GM: Genetics of craniofacial development and malformation. *Nat Rev Genet* 2001;2:458–468.
- Stanier P, Moore GE: Genetics of cleft lip and palate: syndromic genes contribute to the incidence of non-syndromic clefts. *Hum Mol Genet* 2004;13:R73–R81.
- Trokovic N, Trokovic R, Mai P, Partanen J: Fgfr1 regulates patterning of the pharyngeal region. *Genes Dev* 2003;17:141–153.
- Rice R, Spencer-Dene B, Connor EC, Gritli-Linde A, McMahon AP, Dickson C, Thesleff I, Rice DP: Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. *J Clin Invest* 2004;113:1692–1700.

- 19 Eswarakumar VP, Horowitz MC, Locklin R, Morriss-Kay GM, Lonai P: A gain-of-function mutation of Fgfr2c demonstrates the roles of this receptor variant in osteogenesis. *Proc Natl Acad Sci USA* 2004;101:12555–12560.
- 20 Trumpp A, Depew MJ, Rubenstein JL, Bishop JM, Martin GR: Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. *Genes Dev* 1999;13:3136–3148.
- 21 Abu-Issa R, Smyth G, Smoak I, Yamamura K, Meyers EN: Fgf8 is required for pharyngeal arch and cardiovascular development in the mouse. *Development* 2002;129:4613–4625.
- 22 Miyaoka Y, Tanaka M, Imamura T, Takada S, Miyajima A: A novel regulatory mechanism for Fgf18 signaling involving cysteine-rich FGF receptor (Cfr) and delta-like protein (Dlk). *Development* 2010;137:159–167.
- 23 Snyder-Warwick AK, Perlyn CA, Pan J, Yu K, Zhang L, Ornitz DM: Analysis of a gain-of-function FGFR2 Crouzon mutation provides evidence of loss of function activity in the etiology of cleft palate. *Proc Natl Acad Sci USA* 2010;107:2515–2520.
- 24 Britto JA, Evans RD, Hayward RD, Jones BM: Toward pathogenesis of Apert cleft palate: FGF, FGFR, and TGF beta genes are differentially expressed in sequential stages of human palatal shelf fusion. *Cleft Palate Craniofac J* 2002;39:332–340.
- 25 Lee S, Crisera CA, Erfani S, Maldonado TS, Lee JJ, Alkasab SL, Longaker MT: Immunolocalization of fibroblast growth factor receptors 1 and 2 in mouse palate development. *Plast Reconstr Surg* 2001;107:1776–1784.
- 26 Porntaveetus T, Oommen S, Sharpe PT, Ohazama A: Expression of Fgf signalling pathway related genes during palatal rugae development in the mouse. *Gene Expr Patterns* 2010;10:193–198.
- 27 Gong SG, Gong TW, Shum L: Identification of markers of the midface. *J Dent Res* 2005;84:69–72.
- 28 Fuchs A, Inthal A, Herrmann D, Cheng S, Nakatomi M, Peters H, Neubuser A: Regulation of Tbx22 during facial and palatal development. *Dev Dyn* 2010;239:2860–2874.
- 29 Albertson RC, Yelick PC: Fgf8 haploinsufficiency results in distinct craniofacial defects in adult zebrafish. *Dev Biol* 2007;306:505–515.
- 30 Zhang X, Ibrahim OA, Olsen SK, Umemori H, Mohammadi M, Ornitz DM: Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. *J Biol Chem* 2006;281:15694–15700.
- 31 Sunmonu NA, Li K, Li JY: Numerous isoforms of Fgf8 reflect its multiple roles in the developing brain. *J Cell Physiol* 2011;226:1722–1726.
- 32 Xu X, Weinstein M, Li C, Naski M, Cohen RI, Ornitz DM, Leder P, Deng C: Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. *Development* 1998;125:753–765.
- 33 Ohuchi H, Hori Y, Yamasaki M, Harada H, Sekine K, Kato S, Itoh N: FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. *Biochem Biophys Res Commun* 2000;277:643–649.
- 34 Haque T, Nakada S, Hamdy RC: A review of FGF18: its expression, signaling pathways and possible functions during embryogenesis and post-natal development. *Histol Histopathol* 2007;22:97–105.
- 35 Charoenchakorn K, Yokomizo T, Rice DP, Honjo T, Matsuzaki K, Shintaku Y, Imai Y, Wakamatsu A, Takahashi S, Ito Y, Takano-Yamamoto T, Thesleff I, Yamamoto M, Yamashiro T: Runx1 is involved in the fusion of the primary and the secondary palatal shelves. *Dev Biol* 2009;326:392–402.
- 36 Matsumura K, Taketomi T, Yoshizaki K, Arai S, Sanui T, Yoshiga D, Yoshimura A, Nakamura S: Sprouty2 controls proliferation of palate mesenchymal cells via fibroblast growth factor signaling. *Biochem Biophys Res Commun* 2011;404:1076–1082.
- 37 Welsh IC, Hagge-Greenberg A, O'Brien TP: A dosage-dependent role for Spry2 in growth and patterning during palate development. *Mech Dev* 2007;124:746–761.
- 38 Dode C, Hardelin JP: Kallmann syndrome: fibroblast growth factor signaling insufficiency? *J Mol Med* 2004;82:725–734.
- 39 Dodé C, Levilliers J, Dupont JM, De Paepe A, Le Dû N, Soussi-Yanicostas N, Coimbra RS, Delmaghani S, Compain-Nouaille S, Baverel F, Pêcheux C, Le Tessier D, Cruaud C, Delpech M, Speleman F, Vermeulen S, Amalfitano A, Bachelot Y, Bouchard P, Cabrol S, Carel JC, Delemar-van de Waal H, Goulet-Salmon B, Kottler ML, Richard O, Sanchez-Franco F, Saura R, Young J, Petit C, Hardelin JP: Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. *Nat Genet* 2003;33:463–465.
- 40 Wilkie AO, Slaney SF, Oldridge M, Poole MD, Ashworth GJ, Hockley AD, Hayward RD, David DJ, Pulleyn LJ, Rutland P: Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. *Nat Genet* 1995;9:165–172.
- 41 Reardon W, Winter RM, Rutland P, Pulleyn LJ, Jones BM, Malcolm S: Mutations in the fibroblast growth factor receptor 2 gene cause Crouzon syndrome. *Nat Genet* 1994;8:98–103.
- 42 Dode C, Fouveaut C, Mortier G, Janssens S, Bertherat J, Mahoudeau J, Kottler ML, Chabrolle C, Gancel A, Francois I, Devriendt K, Wolczynski S, Pugeat M, Pineiro-Garcia A, Murat A, Bouchard P, Young J, Delpech M, Hardelin JP: Novel FGFR1 sequence variants in Kallmann syndrome, and genetic evidence that the FGFR1c isoform is required in olfactory bulb and palate morphogenesis. *Hum Mutat* 2007;28:97–98.
- 43 Kreiborg S, Cohen MM Jr: The oral manifestations of Apert syndrome. *J Craniofac Genet Dev Biol* 1992;12:41–48.
- 44 Stoler JM, Rosen H, Desai U, Mulliken JB, Meara JG, Rogers GF: Cleft palate in Pfeiffer syndrome. *J Craniofac Surg* 2009;20:1375–1377.
- 45 Braybrook C, Doudney K, Marcano AC, Arnason A, Bjornsson A, Patton MA, Goodfellow PJ, Moore GE, Stanier P: The T-box transcription factor gene TBX22 is mutated in X-linked cleft palate and ankyloglossia. *Nat Genet* 2001;29:179–83.

- 46 Jezewski PA, Vieira AR, Nishimura C, Ludwig B, Johnson M, O'Brien SE, Daack-Hirsch S, Schultz RE, Weber A, Nepomucena B, Romitti PA, Christensen K, Orioli IM, Castilla EE, Machida J, Natsume N, Murray JC: Complete sequencing shows a role for MSX1 in non-syndromic cleft lip and palate. *J Med Genet* 2003;40:399–407.
- 47 Zucchero TM, Cooper ME, Maher BS, ack-Hirsch S, Nepomuceno B, Ribeiro L, Caprau D, Christensen K, Suzuki Y, Machida J, Natsume N, Yoshiura K, Vieira AR, Orioli IM, Castilla EE, Moreno L, Arcos-Burgos M, Lidral AC, Field LL, Liu YE, Ray A, Goldstein TH, Schultz RE, Shi M, Johnson MK, Kondo S, Schutte BC, Marazita ML, Murray JC: Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. *N Engl J Med* 2004;351:769–780.
- 48 Riley BM, Mansilla MA, Ma J, Daack-Hirsch S, Maher BS, Raffensperger LM, Russo ET, Vieira AR, Dode C, Mohammadi M, Marazita ML, Murray JC: Impaired FGF signaling contributes to cleft lip and palate. *Proc Natl Acad Sci USA* 2007;104:4512–4517.
- 49 Riley BM, Murray JC: Sequence evaluation of FGF and FGFR gene conserved non-coding elements in non-syndromic cleft lip and palate cases. *Am J Med Genet A* 2007;143A:3228–3234.

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Wnt Signaling in Lip and Palate Development

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Abstract

Wnt signaling regulates a variety of cell behaviors and represents a major pathway in development and disease. Mutations in Wnt genes and their downstream targets have been implicated in human craniofacial abnormalities, including the most prevalent birth defect, cleft lip with or without palate. Formation of the upper lip and palate is a complicated process and is composed of a series of highly coordinated steps during tissue morphogenesis, which are rigorously controlled by genetic networks. While genetic controls of lip/palate development have been extensively studied, the roles of Wnt signaling in these processes remained poorly understood. Within the cell, Wnt signaling is transduced in a β -catenin-dependent (canonical) or -independent (non-canonical) fashion. Recent studies have demonstrated that the canonical and non-canonical pathways play differential roles but both are essential in lip/palate development. Here we review these studies that have substantially advanced our knowledge by elucidating the function of Wnt signaling in upper lip formation, secondary palate development and their disease settings. These advances are important to delineate the genetic networks controlling craniofacial development and to develop personalized therapeutic strategies in related human birth defects in the future.

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Craniofacial development is regulated by multiple signaling pathways, which precisely control gene expression, cell behavior and pattern formation.

Wnt signaling has been shown to play critical roles in craniofacial morphogenesis, as evidenced by mutations in WNT genes being associated with a number of human birth defects, including cleft lip and/or palate (CLP) [1, 2], and by recent studies using genetically modified mouse models. Binding of Wnt ligands to transmembrane receptors engages several intracellular signaling transductions, therefore signaling can be categorized into two pathways: canonical and non-canonical. In this chapter, we will first summarize known components and signal transduction in these two pathways. We will then discuss mutations of Wnt signaling components and their association with human CLP diseases. Lastly, we will describe expression patterns of both pathways in craniofacial tissues and recent findings about their roles in lip/palate development.

Wnt Signaling: Canonical or Non-Canonical?

In mammals, Wnt genes encode a family of 19 sequence-related growth factors related to the *wingless* gene in *Drosophila*. The first Wnt gene identified in mice was proto-oncogene *int-1* (*integration-1*), found to be the homolog of *Drosophila wingless* (*wg*) [3]. Fusion of these two terms then yields currently ‘Wnt’, which refers to the extracellular ligands of Wnt

signaling [4]. Wnt genes are implicated in embryonic development and adult tissue homeostasis by engaging canonical or non-canonical pathways [1, 5–10].

The canonical pathway stabilizes cytoplasmic β -catenin and facilitates its translocation into the nucleus to regulate target gene expression. It is thus also named the Wnt/ β -catenin pathway. To activate this pathway, Wnt ligands need to bind to both the transmembrane receptor Frizzled (Fzd) and the co-receptors LRP5/6 (Low-density lipoprotein-Related Protein 5 or 6). This binding induces phosphorylation of LRP5/6 intracellular domains, which in turn facilitates binding of LRP to a combination of cytoplasmic mediators, including Dishevelled (Dsh), Axin, Glycogen synthase kinase 3 (Gsk3), Adenomatous Polyposis Coli (APC), and casein kinase I (CK1). In the absence of Wnt canonical signaling, these mediators form a destruction complex to bind β -catenin and facilitate its degradation in the cytoplasm. When Wnt ligands bind to LRP5/6 and Fzd receptor, Dsh, Axin and Gsk3 proteins are recruited to the cell membrane and result in disruption of the β -catenin destruction complex. Consequently, β -catenin accumulates in the cytoplasm and is then translocated into the nucleus, where it binds to lymphoid enhancer-binding factor 1/T cell-specific transcription factor (Lef/Tcf) family members to regulate target genes expression [6, 7, 11].

Besides the canonical pathway, Wnt ligands can also function in a fashion independent of β -catenin. The non-canonical pathways consist of two main branches: the planar cell polarity (PCP) pathway and the Wnt/ Ca^{2+} pathway [9, 12, 13]. The PCP pathway is mediated by the small GTPase Rho and Cdc42 to c-Jun N-terminal kinase (JNK) [14]. In this pathway, Fzd receptors interact with Vangl1/2 receptors to direct cell polarity, but LRP5/6 co-receptors are not required [15]. In addition, the Wnt/ β -catenin pathway requires all three domains present in Dsh: DIX, PDZ and DEP domain, while the PCP pathway only needs the DEP domain [14, 16]. The Wnt/

Ca^{2+} pathway triggers intracellular Ca^{2+} release to activate protein kinase C (PKC) and Ca^{2+} /calmodulin-dependent kinase II (CamKII) [17–20]. Consequently, CamKII activates the mitogen-activated protein kinase (MAPK) that phosphorylates TCF and prevents the β -catenin-TCF/LEF complex from binding DNA, thus inhibiting transcriptional activity of β -catenin-TCF/LEF complex [21].

According to the signaling cascade they engage, Wnt ligands are categorized into canonical and non-canonical Wnts [22]. Wnt1, Wnt3a and Wnt8 are canonical as they act via a β -catenin-dependent canonical pathway, whilst Wnt5a and Wnt11 are assigned as non-canonical, since they activate PCP and Wnt/ Ca^{2+} pathways or act via other receptors, such as Ror2 and Ryk [9, 23–25]. Fzd receptors are also classified into canonical and non-canonical receptors. It is noteworthy that Wnt5a can activate both canonical and non-canonical signaling, depending on certain receptors [26]. Therefore, activation of specific Wnt signaling is determined not only by the ligand but also by the receptor.

Wnt signaling activity is controlled and tuned by multiple factors, including secreted, membranous and intracellular regulators. At the extracellular level, secreted frizzled-related receptor proteins (SFRPs) and WIF1 (Wnt inhibitory factor 1) share similar extracellular domains with Fzd receptors. SFRPs and WIF1 inhibit both canonical and non-canonical signaling by competing for binding of Wnt ligands with their receptors [27]. Wise (also known as USAG-1, Sostdc1 and Ectodin) is an established antagonist of both Wnt and Bmp signaling. It binds to the extracellular domain of LRP5/6 to inhibit Wnt canonical signaling [28]. Dickkopf (Dkk) family members bind to LRP5/6 co-receptors and specifically block canonical signaling [27]. The R-spondins (Rspo) are a newly identified family of Wnt canonical signaling agonists and they function to disrupt LRP6/Dkk1 interaction [29, 30]. At the cell membrane level, LRP4 negatively modulates

LRP5/6-activated canonical signaling [31, 32]. At the intracellular level, there are naked cuticle (NKD) and nemo-like kinase (NLK) to inhibit the canonical signaling [33, 34].

Mutations in WNT Signaling Components Are Associated with CLP in Humans

Accumulated evidence has shown that mutations in WNT signaling genes are highly relevant to human CLP [1, 2, 35]. Single-nucleotide polymorphisms in *WNT3*, *WNT3A*, *WNT5A*, *WNT7A*, *WNT8A*, *WNT9B* and *WNT11* have been linked to non-syndromic cleft lip with or without cleft palate (NSCLP) in European-American populations [1, 2]. Three of them, *WNT3A*, *WNT5A* and *WNT11*, show significant association with NSCLP. Homozygous mutation of *WNT3* causes tetra-amelia syndrome, a rare autosomal recessive birth defect characterized by absence of all four limbs and severe craniofacial defects [36]. *WNT5A* mutation has been linked to human cleft palate in two independent reports [37, 38]. In one of these, mutations in both *WNT5A* and its receptor *ROR2* have been identified in autosomal dominant Robinow syndrome, which causes a spectrum of birth defects including cleft palate (CP) [37]. In the other case the *WNT5A* receptor *RYK* has also been linked to NSCLP [39]. All of these WNT ligands function via canonical or non-canonical signaling to regulate human craniofacial development, but their functional mechanisms remain largely unknown.

Wnt Signaling and Upper Lip Formation

During early mammalian embryogenesis, the face is composed of five prominences: the frontonasal prominence, two maxillary processes and two mandibular processes. The frontonasal processes later differentiate into a medial nasal process in the middle and two lateral nasal processes

at each side. The medial nasal, lateral nasal and maxillary processes fuse at the distal end to form the upper lip. The medial nasal process and two maxillary processes contribute to the palate [40]. In the primordial oral-nasal cavity, the medial nasal process gives rise to the primary palate, and the maxillary processes contribute to the bilateral secondary palate. The secondary palatal shelves initially reside in a vertical orientation besides the developing tongue. Subsequently, they elevate to a horizontal position, meet and fuse at the MEE (Medial Edge Epithelia) to form an intact secondary palatal shelf. The fused secondary palatal shelf then extends anteriorly and fuses with the primary palate to form a functional palate shelf, separating the nasal cavity and oral cavity [41].

Canonical Wnt Signaling in Lip Development

Canonical Wnt signaling plays an essential role in lip and palate development, evidenced by intense expression in these regions and the fact that mutation of key components leads to severe craniofacial defects [5]. Using Wnt reporter transgenic mice, researchers have documented a detailed expression profile for canonical signaling activity in the developing mouse embryo [42, 43]. During early craniofacial morphogenesis, Wnt canonical signaling is prominently expressed in neural tissues and migrating cranial neural crest (CNC) cells that contribute to most mesenchymal cells of the facial prominences [42, 43]. During lip formation, Wnt canonical signaling is expressed in the ectoderm and underlying mesenchyme of the fusing primordia [43]. Loss of *LRP6* significantly attenuates Wnt canonical signaling activity in mutant embryos, as well as cell proliferation. More importantly, the mutant lip primordia fail to fuse due to an absence of cell apoptosis that is required for normal upper lip formation. The altered expression of *Msx2* and *Raldh3* in the absence of *LRP6* could also contribute to the cleft lip defect [44]. *Rspo2* is a canonical signaling agonist genetically interacting with *LRP6*. Consistent to

the central role of LRP6 in upper lip morphogenesis, deletion of *Rspo2* leads to a cleft lip defect [45]. It has been shown that *Rspo2* and LRP6 play synergistic roles in mandible formation and it is likely that such a scenario also exists in upper lip development. This idea is supported by evidence that downregulated expression of *Msx1* and *Msx2* is observed in the frontonasal prominences in both *Rspo2* mutant and *LRP6* mutant embryos [44, 45]. Interestingly, ectopic Wnt canonical signaling in early facial ectoderm also causes a cleft lip, accompanying severe malformation of other craniofacial structures [46]. In summary, these studies suggest a requirement of well-tuned Wnt canonical signaling activity in upper lip formation.

Non-Canonical Wnt Signaling in Lip Formation

The function of non-canonical Wnt signaling in upper lip formation remains to be elucidated. It was reported that *Wnt9b* is expressed in ectoderm of the fusing lip primordia and deletion of *Wnt9b* causes cleft lip [43, 47]. Although it has been suggested that *Wnt9b* might function through the canonical pathway, as its expression pattern overlaps with Wnt canonical signaling reporter activity during lip formation [43], a recent study has revealed that *Wnt9b* induces kidney tubules and regulates nephron morphogenesis via PCP signaling [48]. It is likely that *Wnt9b* regulates lip formation using the same mechanism.

Canonical Wnt Signaling and Palate Development

Although mutations in WNT genes have been linked to CP in humans, the role for Wnt canonical signaling in palatogenesis did not begin to be elucidated until recently. This is partially due to the essential role of canonical signaling in embryogenesis and its absence usually leads to early embryonic lethality or severe abnormalities, making

it difficult to study craniofacial morphogenesis. For example, deletion of the mouse *Catnb* gene, which encodes the Wnt canonical signaling central mediator β -catenin, causes embryonic death before E9.5 [49], and inactivation of *Catnb* from CNCs leads to absence of head structures [50]. Tissue-specific deletion of *Catnb* from facial ectoderm in early mouse embryos results in severely malformed craniofacial structures, preventing an assessment of palate development [46, 51]. It has been reported that a number of components and modulators of Wnt canonical signaling, including *Wnt2*, *Wnt3*, *Wnt4*, *Fzd6*, *Catnb*, *Dkk1*, *LRP5/6*, *Gsk3 α/β* , and *Axin2*, are extensively expressed in palatal epithelium, while the antagonists *Sfrp2* and *Sfrp4* are expressed in the mesenchyme [52–54]. Tissue-specific removal of *Catnb* from palatal epithelial cells generates a persistent MES (medial edge seam), which should have undergone programmed cell death to facilitate palate fusion. As a result, the palatal shelves of *Catnb*^{E/F}; *K14Cre* mice failed to fuse and develop complete clefting [52]. Consistently, deletion of *TCF4* and *LEF1*, two important transcriptional factors of Wnt canonical signaling, also leads to MES persistence [55]. The other gene known to play such a role in palate fusion is *Tgfb β 3*, whose expression overlaps with that of *Catnb* in the palate MEE [56–58], and downregulated *Tgfb β 3* expression is observed in the MEE of *Catnb*^{E/F}; *K14Cre* mice [52]. In addition, it has been reported that β -catenin directly binds to the *Tgfb β 3* promoter [59]. Together, these results indicate that Wnt canonical signaling regulates palate fusion through *Tgfb β 3* transcription. Consistent with the essential role for Wnt canonical signaling in palatogenesis, inactivation of *LRP6* or *Rspo2* also results in palate clefting, but the etiology in these mutants is not well understood. Although it has been proposed that the formation of CP in the *Rspo2* mutant is secondary to the malformed structures adjacent to palate. *Rspo2* is expressed in developing palate mesenchyme, in a restrictive pattern overlapping with *LRP6* (fig. 1), arguing an intrinsic role

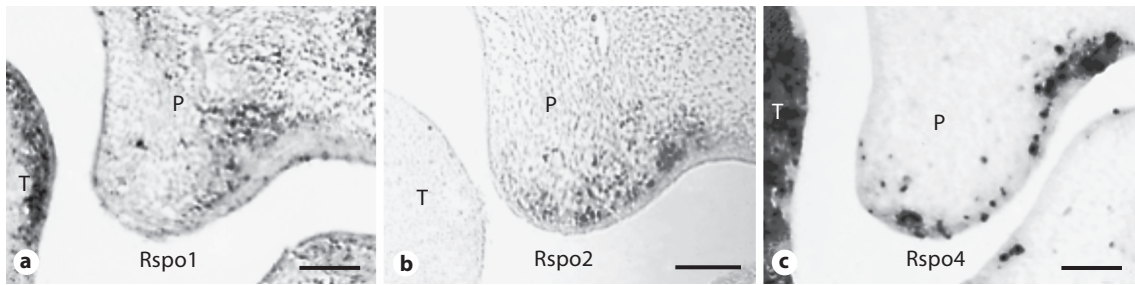


Fig. 1. Expression of Rspo family members in the coronal sections of mouse E13.5 palate. **a** In the E13.5 palatal shelf, Rspo1 is expressed in the lateral palatal mesenchyme and the tongue mesenchyme. **b** Expression of Rspo2 is restricted to the mesenchyme underneath MEE. **c** Rspo4 transcripts are detected in the oral side palatal epithelium and the developing tongue. Rspo3 expression is not detected in the palate. P = Palate; T = tongue. Scale bars = 200 μ m.

for *Rspo2* in palate development [52]. It is intriguing to postulate that similar fusion defects would be observed in *LRP6* and *Rspo2* knockout mice [44, 45].

Wnt canonical signaling is required for the induction and patterning of palate rugae, which are ectodermal appendages that are sequentially formed, serving as a boundary marker and signaling center for palate extension and patterning along the anterior-posterior axis [60, 61]. Palate rugae express *Shh* that is essential for palate development [62, 63]. Wnt canonical signaling is detected in the developing rugae by *LacZ* expression of the Wnt reporter *BATGAL* mice [52, 64]. Either deletion or activation of Wnt canonical signaling in the palatal epithelium causes malformed palate rugae [52, 65]. Disappearance of palate rugae is observed in both *Catnb^{F/F};K14Cre* and *Catnb^{F/F};Shh^{Cregfp}* mice [52, 65]. In contrast, a complete CP is observed in the former and an anterior-only cleft in the latter. Such diversity might arise from the differential expression pattern of Cre recombinase in *K14Cre* and *Shh^{Cregfp}* mice. The *K14Cre* line drives Cre expression in the entire oronasal epithelia while the *Shh^{Cregfp}* expresses Cre only in the rugae and the MEE in the anterior palate [52, 65]. Another difference is that down-regulated *Shh* expression is exclusively found in *Catnb^{F/F};Shh^{Cregfp}* palatal shelves but not in the

Catnb^{F/F};K14Cre palate. This variation might reflect a combination of the hypomorphism of *Shh* expression in *Shh^{Cregfp}* mice and possible technical problems. Expression of stabilized β -catenin in the palatal epithelium also disrupts palatal rugae formation in *Catnb^{F(ex3)};K14Cre* mice [52]. This result is consistent with the report that a disorganized rugae pattern was observed in mice mutant for the Wnt canonical signaling antagonists *Wise* and *LRP4* [32, 61, 66]. Thus, Wnt canonical signaling appears to regulate rugae formation in a dose-sensitive manner.

A proper level of Wnt canonical signaling is stringently required for palate development. Whilst deficient Wnt canonical signaling causes a cleft palate defect, excessive Wnt canonical signals also impair normal palate development. In *Catnb^{F(ex3)};K14Cre* mice, the palatal shelves fail to elevate to a horizontal orientation, leading to cleft palate [52]. Detailed analysis revealed that excessive Wnt canonical signaling induces ectopic *Tgfb β 3* expression in the oral side palatal epithelium and results in abnormal palate-mandible fusion, preventing the mutant palatal shelves from elevating [52]. A palate elevation defect is also observed in *Gsk3 β* knockout mice [53, 67]. Since *Gsk3 β* contributes to the β -catenin destruction complex and represses Wnt canonical signaling, in theory, inactivating *Gsk3 β* should

enhance Wnt canonical signals in the developing palate. Surprisingly, it was found that *Gsk3 β* mutants and *Catnb^{F(ex3)};K14Cre* mice do not share a similar mechanism in regulating palate elevation [53]. Expression of *Axin2*, the Wnt canonical signaling target, was not elevated in the *Gsk3 β* mutant palate and no palate-mandible fusion was observed. In addition, it was discovered that *Gsk3 α* , which functions redundantly with *Gsk3 β* in modulating β -catenin degradation, is co-expressed with *Gsk3 β* during Wnt canonical signaling in palatal epithelium. The unaltered *Gsk3 α* expression could explain normal Wnt canonical signaling in the *Gsk3 β* mutant palate. In support of this explanation, ectopic *Axin2* expression was detected in the *Gsk3 β* mutant tongue mesenchyme, where *Gsk3 α* is absent [53]. These results suggest that *Gsk3 β* functions via a β -catenin-independent way in regulating palate development. It is expected that ablation of both *Gsk3 α* and *Gsk3 β* in the palatal epithelium would phenocopy the cleft palate defect in *Catnb^{F(ex3)};K14Cre* mice. Interestingly, deletion of *Catnb* and expression of stabilized β -catenin in palatal mesenchyme causes CP, respectively [68]. Because Wnt canonical signaling activity is barely detected in the palate mesenchyme, this phenotype might reflect an importance for β -catenin as a cell adhesion molecule and a component of the cytoskeleton, rather than as a Wnt canonical signaling mediator [52, 68].

Non-Canonical Wnt Signaling in Palate Development

Wnt5a is well known to activate non-canonical Wnt signaling [23, 69–71]. *Wnt5a* mutant exhibit a complete cleft palate accompanied by shortened snout and mandible [54, 72]. In the developing palate, *Wnt5a* expression is restricted in the mesenchyme, in a gradient pattern decreasing from anterior to posterior [54]. This pattern indicates that Wnt5a plays a role in anterior-

posterior patterning of the polarized palate. In fact, *Wnt5a* plays differential roles in the anterior and posterior palate in regulating mesenchymal cell proliferation. In the anterior palate, loss of *Wnt5a* caused elevated cell proliferation; whilst cell proliferation level is significantly reduced in the posterior palate mesenchyme [54]. Previous studies have shown that Wnt5a can signal through *Fzd4*, *Ror2* and *Ryk*. *Fzd4* and *Ror2* transcripts are expressed in the developing palate in a gradient manner similar to that of *Wnt5a*, suggesting both receptors may mediate Wnt5a function in regulating palate development [54]. However, the functional importance of *Fzd4* in palate development is questioned by the fact that *Fzd4* knockout mice do not exhibit craniofacial defects and are able to survive to adulthood [73]. However, *Ror2* mutant mice resemble the *Wnt5a* mutant phenotype, exhibiting severe defects in tissue outgrowth as well as a CP [74, 75]. *Wnt5a* and *Ror2* double heterozygous mice develop CP, indicating that *Wnt5a* and *Ror2* play an epistatic role in regulating palate development. In accordance with this phenotypic resemblance, downregulation of Shh signaling is observed in the palatal shelves of *Wnt5a^{-/-}*, *Ror2^{-/-}*, and *Wnt5a^{+/-};Ror2^{+/-}* mice, respectively [54].

In the *Wnt5a* mutant palate, a subgroup of mesenchymal cells expressing *Sox9* exhibit altered distribution. This phenotype correlates with the function of Wnt5a in regulating cell migration in other biological systems [76–79]. In vitro assays revealed that in the normal developing palate, there exist two streams of directionally migrating mesenchymal cells: one migrates along the axis from the posterior towards the anterior end and one from the medial to the lateral region, with Wnt5a serving as an essential chemoattractant for cells migrating along the anterior-posterior axis [54]. In the palatal shelves of both *Wnt5a* and *Ror2* mutants, cell migration from posterior to anterior is disrupted. In addition, exogenous Wnt5a protein failed to chemoattract migration of mesenchymal cells in the *Ror2* mutant palate,

indicating an essential role for Ror2 in mediating Wnt5a regulated directional cell migration during palate development. In some cell contexts, the Wnt5a-Ror2 pathway has been shown to antagonize Wnt canonical signaling [26]. However, ectopic Wnt canonical signaling activity was not observed in the *Wnt5a* mutant palate, suggesting that Wnt5a-Ror2 signaling functions other than by inhibiting the Wnt canonical pathway [54]. It is also noteworthy that inactivation of *Ryk*, which encodes another Wnt5a receptor, also causes cleft palate [80]. Wnt5a exerts a chemorepulsive property for neuronal cells expressing *Ryk* [81]. It is thus intriguing to postulate that Wnt5a engages both Ror2 and Ryk in directional palatal cell migration.

Wnt11 is expressed exclusively in MEE cells of the developing palate, suggesting a role in palate fusion [82]. Indeed, results from in vitro assays have shown that knockdown of *Wnt11* leads to persistent MES and prevents palate fusion [82]. *Wnt11* knockout mice show perinatal death [83], a characteristic of mice bearing a CP defect. It is thus very likely that *Wnt11* knockout mice carry a CP. Interestingly, it was reported that *Wnt11* knockout mice show excessive β -catenin accumulation in cardiomyocytes, suggesting that *Wnt11* might be involved in regulating palate development via repressing canonical Wnt signaling [84].

PCP signaling has also been implicated in palate development. Fzd2 interacts with the Vangl2

receptor and *Fzd2* mutant mice develop a cleft palate with low penetrance [85]. *Fzd2* and *Fzd1* double knockout mice show cleft palate with complete penetrance. Further analysis revealed ectopic cell apoptosis in the mutant palate epithelium, suggesting that PCP signaling plays a role in controlling palatal epithelial cell survival [85].

Both canonical and non-canonical Wnt signaling pathways are involved in the regulation of cell proliferation, survival, migration and patterning in palate development. However, we are still far away from a complete understanding of the roles and functional mechanisms in each step of palatogenesis. It is still not known if cross-talk occurs between canonical and non-canonical Wnt signaling pathways or other signaling pathways. A combination of newly developed high-throughput technologies with traditional genetic, molecular, and experimental embryology approaches will aid advance in elucidation of the morphogenetic and molecular mechanisms of palatogenesis and cleft palate formation.

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References

- 1 Chiquet BT, Blanton SH, Burt A, Ma D, Stal S, Mulliken JB, Hecht JT: Variation in WNT genes is associated with non-syndromic cleft lip with or without cleft palate. *Hum Mol Genet* 2008;17:2212–2218.
- 2 Menezes R, Letra A, Kim AH, Kuchler EC, Day A, Tannure PN, Gomes da Motta L, Paiva KB, Granjeiro JM, Vieira AR: Studies with Wnt genes and nonsyndromic cleft lip and palate. *Birth Defects Res A Clin Mol Teratol* 2010;88:995–1000.
- 3 Nusse R, Varmus HE: Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* 1982;31:99–109.

- 4 Nusse R, Brown A, Papkoff J, Scambler P, Shackleford G, McMahon A, Moon R, Varmus H, Smolich BD, McMahon JA: A new nomenclature for int-1 and related genes: the Wnt gene family. *Cell* 1991;64:231.
- 5 Liu F, Millar SE: Wnt/ β -catenin signaling in oral tissue development and disease. *J Dent Res* 2010;89:318–330.
- 6 Klaus A, Birchmeier W: Wnt signalling and its impact on development and cancer. *Nat Rev Cancer* 2008;8:387–398.
- 7 Clevers H: Wnt/ β -catenin signaling in development and disease. *Cell* 2006;127:469–480.
- 8 Logan CY, Nusse R: The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 2004;20:781–810.
- 9 Veeman MT, Axelrod JD, Moon RT: A second canon: functions and mechanisms of β -catenin-independent Wnt signaling. *Dev Cell* 2003;5:367–377.
- 10 Nelson WJ, Nusse R: Convergence of Wnt, β -catenin, and cadherin pathways. *Science* 2004;303:1483–1487.
- 11 Grigoryan T, Wend P, Klaus A, Birchmeier W: Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of β -catenin in mice. *Genes Dev* 2008;22:2308–2341.
- 12 Sugimura R, Li L: Noncanonical Wnt signaling in vertebrate development, stem cells, and diseases. *Birth Defects Res C Embryo Today* 2010;90:243–256.
- 13 Pandur P: Recent discoveries in vertebrate non-canonical Wnt signaling: towards a Wnt signaling network; in Marek M (ed): *Advances in Developmental Biology*. New York, Elsevier, 2005, pp 91–106.
- 14 Mlodzik M: Planar cell polarization: do the same mechanisms regulate *Drosophila* tissue polarity and vertebrate gastrulation? *Trends Genet* 2002;18:564–571.
- 15 Wansleben C, Meijlink F: The planar cell polarity pathway in vertebrate development. *Dev Dyn* 2011;240:616–626.
- 16 Church VL, Francis-West P: Wnt signaling during limb development. *Int J Dev Biol* 2002;46:927–936.
- 17 Kuhl M, Sheldahl LC, Malbon CC, Moon RT: Ca^{2+} /calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in *Xenopus*. *J Biol Chem* 2000;275:12701–12711.
- 18 Kuhl M, Sheldahl LC, Park M, Miller JR, Moon RT: The Wnt/ Ca^{2+} pathway: a new vertebrate Wnt signaling pathway takes shape. *Trends Genet* 2000;16:279–283.
- 19 Slusarski DC, Corces VG, Moon RT: Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. *Nature* 1997;390:410–413.
- 20 Slusarski DC, Yang-Snyder J, Busa WB, Moon RT: Modulation of embryonic intracellular Ca^{2+} signaling by Wnt-5A. *Dev Biol* 1997;182:114–120.
- 21 Ishitani T, Kishida S, Hyodo-Miura J, Ueno N, Yasuda J, Waterman M, Shibuya H, Moon RT, Ninomiya-Tsuji J, Matsumoto K: The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/ Ca^{2+} pathway to antagonize Wnt/ β -catenin signaling. *Mol Cell Biol* 2003;23:131–139.
- 22 Miller JR: The Wnts. *Genome Biol* 2001;3:3001–3015.
- 23 Topol L, Jiang X, Choi H, Garrett-Beal L, Carolan PJ, Yang Y: Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent β -catenin degradation. *J Cell Biol* 2003;162:899–908.
- 24 Wallingford JB, Mitchell B: Strange as it may seem: the many links between Wnt signaling, planar cell polarity, and cilia. *Genes Dev* 2011;25:201.
- 25 Van Amerongen R, Mikels A, Nusse R: Alternative Wnt signaling is initiated by distinct receptors. *Sci Signal* 2008;1:re9.
- 26 Mikels AJ, Nusse R: Purified Wnt5a protein activates or inhibits β -catenin-TCF signaling depending on receptor context. *PLoS Biol* 2006;4:e115.
- 27 Kawano Y, Kypta R: Secreted antagonists of the Wnt signalling pathway. *J Cell Sci* 2003;116:2627–2634.
- 28 Laurikkala J, Kassai Y, Pakkasjärvi L, Thesleff I, Itoh N: Identification of a secreted BMP antagonist, ectodin, integrating BMP, FGF, and SHH signals from the tooth enamel knot. *Dev Biol* 2003;264:91–105.
- 29 Kazanskaya O, Glinka A, del Barco Barantes I, Stanek P, Niehrs C, Wu W: R-spondin2 is a secreted activator of Wnt/ β -catenin signaling and is required for *Xenopus* myogenesis. *Dev Cell* 2004;7:525–534.
- 30 Kim K-A, Wagle M, Tran K, Zhan X, Dixon MA, Liu S, Gros D, Korver W, Yonkovich S, Tomasevic N, Binnerts M, Abo A: R-spondin family members regulate the Wnt pathway by a common mechanism. *Mol Biol Cell* 2008;19:2588–2596.
- 31 Johnson EB, Hammer RE, Herz J: Abnormal development of the apical ectodermal ridge and polysyndactyly in *Megf7*-deficient mice. *Hum Mol Genet* 2005;14:3523.
- 32 Ohazama A, Johnson EB, Ota MS, Choi HJ, Porntaveetus T, Oommen S, Itoh N, Eto K, Gritli-Linde A, Herz J: Lrp4 modulates extracellular integration of cell signaling pathways in development. *PLoS One* 2008;3:e4092.
- 33 Wharton K: Vertebrate proteins related to *Drosophila* naked cuticle bind dishevelled and antagonize Wnt signaling. *Dev Biol* 2001;234:93–106.
- 34 Ishitani T, Ninomiya-Tsuji J, Nagai S, Nishita M, Meneghini M, Barker N, Waterman M, Bowerman B, Clevers H, Shibuya H: The TAK1-NLK-MAPK-related pathway antagonizes signalling between β -catenin and transcription factor TCF. *Nature* 1999;399:798–802.
- 35 Carinci P, Becchetti E, Baroni T, Carinci F, Pezzetti F, Stabellini G, Locci P, Scapoli L, Tognon M, Volinia S, Bodo M: Extracellular matrix and growth factors in the pathogenesis of some craniofacial malformations. *Eur J Histochem* 2007;51(suppl 1):105–115.
- 36 Niemann S, Zhao C, Pascu F, Stahl U, Aulepp U, Niswander L, Weber J, Muller U: Homozygous WNT3 mutation causes tetra-amelia in a large consanguineous family. *Am J Hum Genet* 2004;74:558–563.
- 37 Person AD, Beiraghi S, Sieben CM, Hermanson S, Neumann AN, Robu ME, Schleiffarth JR, Billington CJ Jr, van Bokhoven H, Hoogeboom JM, Mazzeul JF, Petryk A, Schimmenti LA, Brunner HG, Ekker SC, Lohr JL: WNT5A mutations in patients with autosomal dominant Robinow syndrome. *Dev Dyn* 2010;239:327–337.

- 38 Blanton SH, Bertin T, Serna M, Stal S, Mulliken JB, Hecht JT: Association of chromosomal regions 3p21.2, 10p13, and 16p13.3 with nonsyndromic cleft lip and palate. *Am J Med Genet A* 2004; 125A:23–27.
- 39 Watanabe A, Akita S, Tin NTD, Natsume N, Nakano Y, Niikawa N, Uchiyama T, Yoshiura K: A mutation in *RYK* is a genetic factor for nonsyndromic cleft lip and palate. *Cleft Palate Craniofac J* 2006;43:310–316.
- 40 Jiang R, Bush JO, Lidral AC: Development of the upper lip: morphogenetic and molecular mechanisms. *Dev Dyn* 2006;235:1152–1166.
- 41 Ferguson MWJ: Palate development. *Development* 1988;103(suppl):41–60.
- 42 Mani P, Jarrell A, Myers J, Atit R: Visualizing canonical Wnt signaling during mouse craniofacial development. *Dev Dyn* 2010;239:354–363.
- 43 Lan Y, Ryan R, Zhang Z, Bullard S, Bush J, Maltby K, Lidral A, Jiang R: Expression of *Wnt9b* and activation of canonical Wnt signaling during midfacial morphogenesis in mice. *Dev Dyn* 2006;235: 1448–1454.
- 44 Song L, Li Y, Wang K, Wang Y-Z, Molotkov A, Gao L, Zhao T, Yamagami T, Wang Y, Gan Q, Pleasure DE, Zhou CJ: *Lrp6*-mediated canonical Wnt signaling is required for lip formation and fusion. *Development* 2009;136:3161–3171.
- 45 Yamada W, Nagao K, Horikoshi K, Fujikura A, Ikeda E, Inagaki Y, Kakitani M, Tomizuka K, Miyazaki H, Suda T, Takubo K: Craniofacial malformation in *R-spondin2* knockout mice. *Biochem Biophys Res Commun* 2009;381:453–458.
- 46 Reid BS, Yang H, Melvin VS, Taketo MM, Williams T: Ectodermal Wnt/ β -catenin signaling shapes the mouse face. *Dev Biol* 2011;349:261–269.
- 47 Carroll TJ, Park J-S, Hayashi S, Majumdar A, McMahon AP: *Wnt9b* Plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev Cell* 2005;9:283–292.
- 48 Karner CM, Chirumamilla R, Aoki S, Igarashi P, Wallingford JB, Carroll TJ: *Wnt9b* signaling regulates planar cell polarity and kidney tubule morphogenesis. *Nat Genet* 2009;41:793–799.
- 49 Huelsken J, Vogel R, Brinkmann V, Erdmann B, Birchmeier C, Birchmeier W: Requirement for β -catenin in anterior-posterior axis formation in mice. *J Cell Biol* 2000;148:567–578.
- 50 Brault V, Moore R, Kutsch S, Ishibashi M, Rowitch DH, McMahon AP, Sommer L, Boussadia O, Kemler R: Inactivation of the β -catenin gene by *Wnt1-Cre*-mediated deletion results in dramatic brain malformation and failure of craniofacial development. *Development* 2001;128:1253–1264.
- 51 Wang Y, Song L, Zhou CJ: The canonical Wnt/ β -catenin signaling pathway regulates Fgf signaling for early facial development. *Dev Biol* 2011;349:250–260.
- 52 He F, Xiong W, Wang Y, Li L, Liu C, Yamagami T, Taketo MM, Zhou C, Chen YP: Epithelial Wnt/ β -catenin signaling regulates palatal shelf fusion through regulation of *Tgfb3* expression. *Dev Biol* 2010;350:511–519.
- 53 He F, Popkie AP, Xiong W, Li L, Wang Y, Phiel CJ, Chen Y: *Gsk3 β* is required in the epithelium for palatal elevation in mice. *Dev Dyn* 2010;239:3235–3246.
- 54 He F, Xiong W, Yu X, Espinoza-Lewis R, Liu C, Gu S, Nishita M, Suzuki K, Yamada G, Minami Y, Chen Y: *Wnt5a* regulates directional cell migration and cell proliferation via *Ror2*-mediated noncanonical pathway in mammalian palate development. *Development* 2008;135:3871–3879.
- 55 Brugmann SA, Goodnough LH, Gregoriuff A, Leucht P, ten Berge D, Fuerer C, Clevers H, Nusse R, Helms JA: Wnt signaling mediates regional specification in the vertebrate face. *Development* 2007;134:3283–3295.
- 56 Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, Groffen J: Abnormal lung development and cleft palate in mice lacking TGF- β 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet* 1995;11:415–421.
- 57 Taya Y, O’Kane S, Ferguson MW: Pathogenesis of cleft palate in TGF- β 3 knockout mice. *Development* 1999;126:3869–3879.
- 58 Proetzel G, Pawlowski SA, Wiles MV, Yin M, Boivin GP, Howles PN, Ding J, Ferguson MWJ, Doetschman T: Transforming growth factor- β 3 is required for secondary palate fusion. *Nat Genet* 1995;11: 409–414.
- 59 Medici D, Hay ED, Olsen BR: Snail and Slug promote epithelial-mesenchymal transition through β -catenin-T-cell factor-4-dependent expression of transforming growth factor- β 3. *Mol Biol Cell* 2008;19:4875–4887.
- 60 Pantalacci S, Prochazka J, Martin A, Rothova M, Lambert A, Bernard L, Charles C, Viriot L, Peterkova R, Laudet V: Patterning of palatal rugae through sequential addition reveals an anterior/posterior boundary in palatal development. *BMC Dev Biol* 2008;8:1–17.
- 61 Welsh IC, O’Brien TP: Signaling integration in the rugae growth zone directs sequential SHH signaling center formation during the rostral outgrowth of the palate. *Dev Biol* 2009;336:53–67.
- 62 Gritli-Linde A: Molecular control of secondary palate development. *Dev Biol* 2007;301:309–326.
- 63 Lan Y, Jiang R: Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth. *Development* 2009;136:1387–1396.
- 64 Maretto S, Cordenonsi M, Dupont S, Braghetta P, Broccoli V, Hassan AB, Volpin D, Bressan GM, Piccolo S: Mapping Wnt/ β -catenin signaling during mouse development and in colorectal tumors. *Proc Natl Acad Sci USA* 2003;100:3299–3304.
- 65 Lin C, Fisher AV, Yin Y, Maruyama T, Veith GM, Dhandha M, Huang GJ, Hsu W, Ma L: The inductive role of Wnt- β -catenin signaling in the formation of oral apparatus. *Dev Biol* 2011;356:40–50.
- 66 Ohazama A, Porntaveetus T, Ota M, Herz J, Sharpe P: *Lrp4*: a novel modulator of extracellular signaling in craniofacial organogenesis. *Am J Med Genet A* 2010;152:2974–2983.
- 67 Liu KJ, Arron JR, Stankunas K, Crabtree GR, Longaker MT: Chemical rescue of cleft palate and midline defects in conditional GSK-3 β mice. *Nature* 2007;446: 79–82.
- 68 Chen J, Lan Y, Baek J-A, Gao Y, Jiang R: Wnt/ β -catenin signaling plays an essential role in activation of odontogenic mesenchyme during early tooth development. *Dev Biol* 2009;334:174–185.

- 69 Torres MA, Yang-Snyder JA, Purcell SM, DeMarais AA, McGrew LL, Moon RT: Activities of the Wnt-1 class of secreted signaling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early *Xenopus* development. *J Cell Biol* 1996;133:1123–1137.
- 70 Kilian B, Mansukoski H, Barbosa FC, Ulrich F, Tada M, Heisenberg CP: The role of Ppt/Wnt5 in regulating cell shape and movement during zebrafish gastrulation. *Mech Dev* 2003;120:467–476.
- 71 Moon RT, Campbell RM, Christian JL, McGrew LL, Shih J, Fraser S: Xwnt-5A: a maternal Wnt that affects morphogenetic movements after overexpression in embryos of *Xenopus laevis*. *Development* 1993;119:97–111.
- 72 Yamaguchi TP, Bradley A, McMahon AP, Jones S: A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development* 1999;126:1211–1223.
- 73 Hsieh M, Boerboom D, Shimada M, Lo Y, Parlow AF, Luhmann UFO, Berger W, Richards JS: Mice null for Frizzled4 (*Fzd4*^{-/-}) are infertile and exhibit impaired corpora lutea formation and function. *Biol Reprod* 2005;73:1135–1146.
- 74 Oishi I, Suzuki H, Onishi N, Takada R, Kani S, Ohkawara B, Koshida I, Suzuki K, Yamada G, Schwabe GC, Mundlos S, Shibuya H, Takada S, Minami Y: The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. *Genes Cells* 2003;8:645–654.
- 75 Schwabe GC, Trepczik B, Suring K, Brieske N, Tucker AS, Sharpe PT, Minami Y, Mundlos S: Ror2 knockout mouse as a model for the developmental pathology of autosomal recessive Robinow syndrome. *Dev Dyn* 2004;229:400–410.
- 76 Weeraratna AT, Jiang Y, Hostetter G, Rosenblatt K, Duray P, Bittner M, Trent JM: Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. *Cancer Cell* 2002;1:279–288.
- 77 Kim HJ, Schleiffarth JR, Jessurun J, Sumanas S, Petryk A, Lin S, Ekker SC: Wnt5 signaling in vertebrate pancreas development. *BMC Biol* 2005;3:23.
- 78 Jopling C, den Hertog J: Fyn/Yes and non-canonical Wnt signalling converge on RhoA in vertebrate gastrulation cell movements. *EMBO Rep* 2005;6:426.
- 79 Witze ES, Litman ES, Argast GM, Moon RT, Ahn NG: Wnt5a control of cell polarity and directional movement by polarized redistribution of adhesion receptors. *Science* 2008;320:365.
- 80 Halford MM, Armes J, Buchert M, Meskenaite V, Grail D, Hibbs ML, Wilks AF, Farlie PG, Newgreen DF, Hovens CM: Ryk-deficient mice exhibit craniofacial defects associated with perturbed Eph receptor crosstalk. *Nat Genet* 2000;25:414–418.
- 81 Keeble TR, Halford MM, Seaman C, Kee N, Macheda M, Anderson RB, Stacker SA, Cooper HM: The Wnt receptor Ryk is required for Wnt5a-mediated axon guidance on the contralateral side of the corpus callosum. *J Neurosci* 2006;26:5840–5848.
- 82 Lee J-M, Kim J-Y, Cho K-W, Lee M-J, Cho S-W, Kwak S, Cai J, Jung H-S: Wnt11/Fgfr1b cross-talk modulates the fate of cells in palate development. *Dev Biol* 2008;314:341–350.
- 83 Majumdar A, Vainio S, Kispert A, McMahon J, McMahon AP: Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development. *Development* 2003;130:3175–3185.
- 84 Abdul-Ghani M, Dufort D, Stiles R, De Repentigny Y, Kothary R, Megeney LA: Wnt11 Promotes cardiomyocyte development by caspase-mediated suppression of canonical Wnt signals. *Mol Cell Biol* 2011;31:163–178.
- 85 Yu H, Smallwood PM, Wang Y, Vidaltamayo R, Reed R, Nathans J: Frizzled 1 and Frizzled 2 genes function in palate, ventricular septum and neural tube closure: general implications for tissue fusion processes. *Development* 2010;137:3707–3717.

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Treatment Outcome for Children Born with Cleft Lip and Palate

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Abstract

In the management of children born with orofacial clefting there is a danger that the information on genetic and environmental influences together with data emerging from randomized controlled trials are divorced from the current reality of clinical care. It is important that treatment outcomes are constantly reviewed as new evidence unfolds but for many children born with a cleft, basic care will be more rapidly improved through examination of quality of outcomes than the higher level scientific exploration of gene-environment interactions and clinical trials. There are good examples of how scrutiny of outcomes has led to changes in service and an improvement of care. These changes have subsequently improved outcomes. These examples will be explored in some depth as well as the outcomes that are seen as relevant to carers and users. Finally the need to determine the influence of genetics and environments on outcomes is seen as the ultimate goal of total care. If we are clear as to how outcomes might be improved for an individual then reference to genetic determinants will provide a bespoke care pathway for surgical interventions, speech and language therapy, psychological and educational support as well as many other areas. This will however require a detailed longitudinal cohort study of all phenotypes to understand the gene-environment interactions, individual development and treatment outcomes. Thus, outcomes can be used to define service and policy, drive scientific investi-

gation, but most importantly improve the care of those children born with a cleft.

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Limitations of Outcome Studies

The long held beliefs by clinicians on strength of evidence have been difficult to inculcate in cleft carers but over the last several decades there is a realization that if care is to be evidence based then the highest levels of research need to be applied. The strongest evidence is clearly derived from randomized controlled trials where confounders are removed by the size of the sample and control for bias through randomization. Randomized controlled trials in the treatment of cleft lip and palate treatment require time, money and large samples. Although clefting is a common congenital anomaly in man (1:700 live births), because of the variation in phenotypic expression it is difficult to collect, from a single centre, sufficient numbers to randomize to specific treatments. Not surprisingly the next level of evidence has proved slightly easier to develop and this involves inter-centre comparisons of outcomes. A number of these studies in cleft lip and palate have been undertaken often involving different countries. This is a recognized way of increasing participant

numbers and has value in that if the results are appropriately interpreted and there is genuine attempt to minimize bias, the findings will be relevant. Controlling bias in these studies is difficult; in particular, overcoming intrinsic differences which can be attributed to the procedures. This can be overcome with a large sample of consecutively treated cases with clear documentation to show the samples are equivalent. The next obvious bias to overcome is follow-up, which can be minimized by the requirement to account for any patients who are enrolled in the study but the centre is unable to make outcome records available. This is inevitable where patients move away or fail to return for team evaluations. In the analysis of records, some bias can be overcome through statistically determined minimal sample sizes and blinding all raters and examiners as to where the records are derived from. Attempts at determining sample sizes for outcome studies are difficult but nevertheless, statistically, for a centre treating 30 new patients with complete unilateral cleft lip and palate (UCLP) it would take 12 years to gather the required number of cases to enter into a five-centre inter-centre study. If only 6 new cases are seen per year, it would require 63 years to have a sufficiently large sample for a five-group versus one-reference study and 42 years for a two-centre study. These calculations are based on a 5% probability and 80% power, detection of a 0.5 Goslon scale (see later) point difference in 10-year-old study models [1].

Training and calibration of all raters and examiners is crucial and should continue throughout the analyses to determine reliability between the raters both within their own ratings and between the different raters. Records and the outcomes they represent are the most immediate and simplest way to initiate an inter-centre collaborative study which can provide key information on service provision and development. It is important to remember that observational cohort studies can produce results similar to those of randomized controlled trials when similar criteria are used to

select study subjects. In addition, data from non-medical research do not support a hierarchy of research designs. Finally, the finding that there is substantial variation in the results of randomized, controlled trials is consistent with prior evidence of contradictory results among randomized, controlled trials [2].

Existing Outcomes

Any outcome measurement needs to be undertaken with assessment tools that are reliable (measure consistently whoever uses it), reproducible (any measurement or assessment is the same if measured for a second time) and valid (measures what it is supposed to measure). Unless these basic tenets are followed, any index or rating of an outcome will be of little value and should be interpreted with caution. Where there is doubt or uncertainty on measurement then multiple tools will be developed, all of which will have their own limitations. Unfortunately, this is true for almost every aspect of outcome measurement in cleft care. As technology changes, these measurement tools will become more sophisticated and hopefully more satisfactory. An example is the evolving technology in 3D and 4D imaging (radiographic and photographic) which has applications for evaluating growth, appearance, bone fill and surgical outcomes. It is worth considering how important the developments of indices are in some detail. This highlights how outcome measures evolve and change over time and with advancements in understanding and science. Surgical outcomes will be used as an example of how indices develop and can be used.

Surgical Outcomes

It has become increasingly important to develop assessment tools that provide a basis on which surgeons can judge their results and relate any changes in technique or timing to outcome. Various methods of record collection have been used to enable assessment of outcome in children

born with a cleft. These include radiographs, photographs and dental study models. The assessment of dentoalveolar relations between the maxilla and mandible is a well-recognized indicator of the effect of primary surgery on the maxilla and facial growth. The full effects of surgery cannot be determined until late adolescence when the majority of facial growth has ceased. The development of a robust and reproducible index to assess dental arch relationships [3] has shown that the detection of differences in outcome as early as 10 years of age is possible [4, 5]. The development of study model outcome measures are described in detail to illustrate the complexity and difficulties in constructing an agreed and consistently used outcome measure.

Study Models

Study models per se have the major advantage that standardization in construction can be easily achieved. They can allow both objective and subjective measurements to be undertaken. A number of methods exist to evaluate the relationship of the maxilla and the mandible and the resulting occlusion using study models. The scores assigned to the study models are consequently related to assessment of surgical outcome in cleft lip and palate [3, 6–8].

An early method of assessing dental arch relationships in cleft lip and palate, described the prevalence and type of crossbite in the deciduous dentition [8]. This system has limitations in that it focuses on crossbites and fails to take into account other, possibly more important, dental features of the occlusion. More widely used systems are available and these are broadly based on a method of assessing dental arch relationships, the Goslon (Great Ormond Street London and Oslo) yardstick [3]. This method of scoring categorizes dental arch relationships in terms of anterior-posterior, vertical and transverse relationships in children with only UCLP. The assessment represents the severity of the malocclusion and the features that pose the greatest difficulties in correcting it. It is

an indicator of maxillary growth and this in turn, reflects impacts of primary surgery and alveolar bone grafting. The Goslon yardstick is a simple subjective tool that uses 22 reference models to rank patient study models into groups from 1 to 5, representing an excellent, good, fair, poor and very poor result, respectively. Generally, groups 1 and 2 have occlusions that require no treatment or require just orthodontics alone. Group 3 require more complex orthodontics, but a good result can still be expected. Patients in group 4 would be at the upper limits of orthodontic correction alone, unless facial growth is unfavourable, in which case orthognathic surgery would also be needed. Cases in group 5 require orthognathic surgery to correct the underlying skeletal malrelationships [3]. The anterior-posterior relationship is considered to be of greatest clinical importance in grading the study models using the Goslon yardstick, with the incisal overjet the most significant predictive measure [9]. However, if overjet is used as the single assessment tool it does not represent the overall dental arch accurately and difficulties may arise when scoring borderline cases. The Goslon yardstick has been shown to be a reliable and reproducible means of measuring dental arch relationships and is capable of discriminating the quality of treatment results between different centres, such as the Eurocleft six-centre international study [4, 5, 10], the Clinical Standards Advisory Group (CSAG) project in the UK [11] and most recently the Americleft study [12–16]. The Goslon yardstick has also been used successfully in local and regional surveys [14, 17–20].

The Goslon yardstick was originally designed for use in the late mixed and early permanent dentition. However, it has also been used as a longitudinal assessment tool for dental arch relationships at all stages of dental development from the deciduous, into the permanent dentition [21]. It has been argued that the Goslon yardstick was not designed for the deciduous dentition and a more appropriate index was required to specifically assess the quality of surgical outcome in the primary

dentition. It was suggested that the development of such a scoring system would also help predict the outcome even earlier and provide a surgeon with a basis on which to judge their results and justify any modifications to technique [6]. In addition, it is thought that early predictors of treatment outcome provide a means to reduce the length of research studies without increasing the sample size [22]. A 5-year-old index was therefore developed, retaining the format of the original Goslon yardstick and using reference models to categorize patient study models born with UCLP from 1 to 5. The main advantage of the index at the age of five is that it is an early predictor for poor techniques or operators and it is not 'confounded' by any orthodontic treatments which may camouflage the underlying skeletal problems.

The Goslon and 5-year-old indices are the most widely used assessments in determining dentoalveolar relations in UCLP. Further refinements include the use of 3D reference models to improve the portability of the 5-year-old index [23] and the development of indices for other phenotypes of clefting, such as in bilateral cleft lip and palate. Three bilateral cleft lip and palate yardsticks for different developmental stages of the dentition have recently been constructed, one for the deciduous dentition (6-year-olds' yardstick), one for early mixed dentition (9-year-olds' yardstick), and one for early permanent dentition (12-year-olds' yardstick) [24]. The additional information on growth, which can be derived from lateral cephalograms has also been recently incorporated into these assessments, such that a statistically significant negative correlation exists between Goslon scores and ANB angle. Hence, as the Goslon score worsens, so does the skeletal maxillary-mandibular relationship in an antero-posterior direction [14].

Lateral Cephalograms

An obvious outcome measure in children born with a cleft is to use lateral cephalograms and make cephalometric comparisons of craniofacial

morphology and soft tissues in patients from cleft/craniofacial centres with varied surgical management protocols [25]. This was one of the first studies where outcomes were used to improve our understanding of potential methods to improve care. There were clear craniofacial morphological differences associated with different care protocols. The study was limited in only assessing craniofacial morphology.

Other Outcomes in Cleft Care

There is a similar scenario for outcomes in all other areas of cleft care as described for growth outcomes. Speech, psychology, educational attainment, quality of life and bone grafting have all developed outcome measures which require development and refinement as detailed for dental study casts and radiographs. These will change as science and technology evolves. The use of outcome studies in Europe has defined cleft care in a wide context. Three outcome studies are now discussed because of the impact these have had on the participating centres and beyond.

Eurocleft

The Eurocleft study [4, 5, 26] undertaken in the 1980s represented an advance in outcome-based studies in that compared to the Ross approach, more detailed and verified information was collected, albeit from only six European centres. The first approach was to conduct essentially an audit of the surgical protocols and approaches to care taken by the different centres. This was relatively straightforward in that each centre was asked to produce records from approximately 25 consecutive treated cases of children born with UCLP. At that time, there were few centres in Europe, which were able to produce these records. A number of outcomes were collected and analysed. The study was important because it compared the outcomes of the 6 centres, rather than the individual patients. The craniofacial morphology (skeletal pattern)

and the facial morphology (soft tissue profile) were evaluated using cephalometric radiographs of the full cohort of 151 UCLP cases from the six centres [27]. Only one centre showed notable and consistent negative differences in skeletal pattern from the others. A contributing factor for these differences was thought to be an inconsistent treatment regimen with many surgeons involved. Analysis of the soft tissue profile between the centres showed more pronounced differences than analysis of the skeletal profile. The treatment outcome in centres with more complex or expensive programs was no better than those centres using simpler management approaches. This raises doubts on the value of skeletal evaluation in cleft lip and palate. Although used by many investigations and over many years, the reliability and validity is questionable, mainly because the anterior part of the maxilla is incomplete and teeth may be missing. The study models of the cohort were more revealing. One hundred and forty-nine dental casts of the subjects with complete unilateral clefts of the lip and palate from six European cleft palate centres were assessed by means of the Goslon yardstick. The yardstick proved capable of discriminating between the quality of the dental arch relationships between the six centres. Two centres showed especially poor results. Three centres obtained satisfactory results although they used different surgical techniques and protocols in each centre. The final centre had results which were variable but not as poor as the two worst centres. One of the centres showing satisfactory dental arch relationships employed a more complex and expensive treatment program than the other two centres, which both used simpler centralized treatment regimens [4]. The soft tissues were further analysed specifically in the highly relevant nasolabial area. One hundred and fifteen frontal and profile photographs of the nasolabial area of subjects with complete unilateral clefts of the lip and palate from six European centres were assessed. Four components of the nasolabial area were rated separately by a panel of judges using a

5-point scale of attractiveness. There were significant differences between the centres. The relative position of the six centres in this study followed a similar pattern to their respective positions with the outcomes derived from study models and cephalometric radiographs [28]. There was also analysis of speech in 131 of the subjects using a specifically designed phonetic framework [29]. This framework focused on consonants that are 'vulnerable' in cleft palate and common to the five languages of the project. The methodology used was complex, but nevertheless set a standard and challenge for future studies. Consonant articulation, resonance, and voice quality were also evaluated. The results showed good outcomes with regard to consonant articulation across the whole study group with common areas of minor difficulty across languages. The results for resonance were less good, with slight hypernasality in 20% of subjects. There were, however, few indications of seriously disordered speech. The detectable differences between centres matched the findings of the other outcomes of the study with regard to the ranking of the centres. This study was important in that it demonstrated that an international, multicentre clinical audit of treatment outcome for complete UCLP cases could discriminate treatment methods and protocols. It also suggested a number of recommendations for the methodology of future studies with respect to entry criteria, sample size, assumptions of homogeneity, and the reproducibility and validity of outcome measures. Although the findings of this study regarding clinical procedures were indicative, it was an important study in setting a challenge to other centres and also the need to improve and extend methodology for future outcome studies. There were differences between centres with high-volume and low-volume operators. The standardization, and the participation of high-volume operators were associated with good outcomes, and non-standardization and the participation of low-volume operators with poor outcomes. Treatment factors associated with good outcomes were the

use of a vomer flap to close the anterior palate. Poor outcomes were associated with primary bone grafting and with active presurgical orthopaedics. It appeared, however, that acceptable results can be achieved by different treatment protocols and ultimately clinical choices may be based on factors such as complexity, costs, and demands of treatment [4, 5, 26–29]. A follow-up of this cohort from five of these centres at age 17 made it clear that the differences between the centres in terms of outcomes still existed [30–34]. Interestingly, there was also some process data made available. The mean number of operations per centre ranged from 3.5 to 6, the length of orthodontic treatment from 3.3 to 8.5 years, and attendance from 49 to 94 visits. This highlighted that protocols for the management of complete UCLP can vary dramatically in the burden of treatment imposed. Comparisons of craniofacial morphology and nasolabial appearance showed that at ages 12 and 17, two centres had a flatter profile and retrognathic maxilla and one of the two centres had increased lower face height. The ratings of nasolabial appearance showed more similarity between the centres. Although these differences were clear, no specific causative factors could be identified. When dental arch relationships were then examined at 17 years of age, three of the centres had better ratings in dental arch relationship than the other two centres at statistically significant levels. The systematic differences in dental arch relationships between different cleft centres could not be ascribed to any specific causative factors. Clearly some outcomes measured in childhood can be predictive over time, but this had been previously reported where study model outcomes at 5 years correlate well with the same individuals at 17 years of age [6]. What was also found in the Eurocleft follow-up was that the amount of treatment does not correlate with the quality of clinical outcome. Interestingly, there was a high level of patient/parent satisfaction and no relationships among satisfaction, objectively rated outcomes, and the amount of care. It seems obvious but the

measurement of clinical outcome in childhood is an important and valid form of clinical audit which can do much to inform policy-makers, governments or healthcare providers.

UK Clinical Standards Advisory Study

In the late 1980s and early 1990s there were indications that care for children born with a cleft lip and palate in the UK was suboptimal [3, 5]. The Department of Health determined that a CSAG enquiry be established to determine national levels of care for cleft patients. The CSAG committee commissioned a research team which attempted to investigate every non-syndromic case of UCLP aged 5 and 12 years throughout the UK. An audit of the process of care (how many operations, operators or what care was available) was undertaken and some key outcomes were measured including speech, hearing, oral health, dentoalveolar and skeletal relations, bone grafting, facial appearance and patient/parent satisfaction. A full account of the methodology is presented elsewhere [35]. The outcomes demonstrated that in key areas there was potentially a need for reorganization. For example, speech was difficult to understand in 19% of 5-year-olds and 4% of 12-year-olds. There was a mild or moderate degree of hearing loss found in 21% of the 5-year-olds and 16% of the 12-year-olds and poor dental arch relationships (Goslon and 5-year-old index groups 4 and 5) were present in 36% of 5-year-olds and 39% of 12-year-olds. This indicates that post-surgical growth disturbance was significant and that nearly 40% of both age groups were likely to need a maxillary osteotomy to correct an underlying skeletal discrepancy between the maxilla and the mandible. Alveolar bone grafting results were also disappointing in that 16% of 12-year-olds had not received a graft, and of those who had a graft, only 58% were successful. Despite the disappointing levels of outcome, the parents and 12-year-old patients were on the whole satisfied with the outcome of care

received [1, 11, 36, 37]. There were significant residual treatment needs in speech therapy, surgery, hearing and dental care. The predominance of low-volume operators and overall poor quality of results limited the exploration of statistical associations between volume and outcome, but nearly 60% of surgeons were dealing with only 1 UCLP case per year [1, 38]. Similar approaches have been adopted to assess process and outcomes of cleft care in Western Australia [39].

Subsequent Changes in Cleft Lip and Palate Service Provision

The biggest impact of this outcome study was that the number of centres offering cleft services in the UK was reduced from 57 to 11 cleft centres or managed clinical networks. This concentration of services provides the opportunity to produce evidence for optimal care through randomized control trials, observational studies, development and evaluation of psychological interventions, genetic studies and qualitative studies of the process of care. There is now an opportunity to re-audit these reconfigured services to determine the impact of this centralization of care and a follow-up study is currently in process. There is some evidence of significant improvements in hard outcomes such as the success of alveolar bone grafting [40–42] and dentoalveolar relations in children treated within a centralised service [43].

Americleft Study

It would seem obvious that a formulaic approach to inter-centre audits had now been established. First the Eurocleft study undertaken in the 1980s showed the powerful information that could be provided through an outcome approach. The more detailed and encompassing CSAG study demonstrated how this information could be used to inform government, change structure, organization and provision of cleft services. The opportunity to develop this further in America was

a significant development. The Americleft study was a North American initiative to undertake an inter-centre outcome study for patients with repaired complete UCLP from five well-established North American cleft centres. The first approach was to conduct essentially a process audit of the surgical protocols and approaches to care by the different centres. By now the value of comparing maxillo-mandibular relationships for individuals with non-syndromic complete UCLP using the Goslon yardstick for dental models had been well established. A total of 169 subjects between 6 and 12 years of age with repaired complete UCLP who were consecutively treated at the five centres were assessed. One centre that incorporated primary alveolar bone grafting showed especially poor Goslon scores that were significantly poorer than the remaining centres. The surgery protocols used by the other four centres did not include primary alveolar bone grafting but involved a number of different lip and palate closure techniques. Using the Goslon yardstick assumptions, the centre with the best scores would be expected to require end-stage maxillary advancement orthognathic surgery in 20% of its patients, whereas the centre with the worst scores would be likely to require this surgery in 66% of its patients. The craniofacial morphology of 148 subjects with repaired complete UCLP who were consecutively treated at four of the five centres was assessed. The centre that was excluded had insufficient records to participate. There were significant differences found for sagittal maxillary prominence among the four centres. Significant differences were seen among the centres for hard and soft tissue maxillary prominence, but not for mandibular prominence, vertical dimensions, or dental inclinations. When the nasolabial aesthetics were examined in the four centres then no statistically significant differences among centres were detected for both total aesthetic scores and for any of the individual aesthetic components. This is perfectly possible as the nasolabial appearance can mask an underlying skeletal and

dental discrepancy. There were no significant differences in nasolabial aesthetics among the centres evaluated and overall good to fair nasolabial aesthetic results were achieved using different treatment protocols. The study has clearly been undertaken in a very different healthcare system and is a very brave move to be assessed by external scrutineers. This information should flow to insurers and most importantly to parents and children. There was less detail on burden of care, oral health, secondary alveolar bone grafting, hearing, speech outcomes, psychosocial assessment by patients and families, patients' evaluation of the treatment they received. These were all detailed in the UK CSAG study and should be an end target rather than the original Eurocleft approach which lacked some of these details. The outcomes from the Americleft study may have highlighted differences in healthcare systems where finance is the predominant driver. Here the outcomes collected may have reflected the difficulties of providing total care in one centre. The outcomes were essentially restricted to measures of craniofacial growth and nasolabial appearance [12–16].

Future Direction for Outcome Studies

The need to involve 'users' of cleft services has become a late recognized priority and a number of organizations now exist to facilitate this process. The James Lind Alliance (JLA) is a non-profit-making initiative funded by the Medical Research Council and the Department of Health that aims to bring together patients and clinicians together in 'Priority Setting Partnerships' to identify and prioritise the unanswered questions that they agree are most important (<http://www.lindalliance.org>). The recognition that clinical trials are only as credible as their outcomes [44] is obvious since clinical interventions are compared by measuring differences in patient outcomes between groups and clinical decisions made on basis of patient

outcomes. Selection of appropriate outcomes for any study is therefore crucial. The 'COMET' (Core Outcome Measures in Effectiveness Trials, <http://www.liv.ac.uk/nwhtmr/comet/comet.htm>) initiative brings together researchers interested in the development and application of agreed standardized sets of outcomes, known as a 'core outcome set'. These sets represent the minimum that should be measured and reported in all clinical trials of a specific condition. They do not imply that outcomes in a particular trial should be restricted to those in the core outcome set. Explicitly there is an expectation that the core outcomes will be collected and reported to allow the results of trials to be compared, contrasted and combined as appropriate, and that researchers will continue to explore development of other outcomes as well. The JLA and COMET initiatives will define developments of outcomes relevant to those children born with a cleft and their families.

The need to fully integrate a growing pool of genetic, environmental and outcome data is self-evident. How all of this information can be pulled into a manageable dataset will require considerable resource and drive. It is crystallized by the two most common questions asked by families with a child born with a cleft: (1) What is the cause? – This requires genetic and environmental information. (2) Will my child be OK? – This requires an understanding of the impact of diagnosis and treatment on clinical outcomes as well as on social and educational development.

Whilst the precise cause of orofacial clefting is unclear, genetic and environmental factors are known to contribute, but to answer these two questions this information needs to be collected through a proposed prospective birth cohort study using a patient-centred approach. This provides a way of linking genetic data (in the form of a gene bank) with environmental information (gleaned from the prospective study) to tease out the relative contribution of each to this common anomaly and its associated outcomes. This type of study will uncover new genetic pathways

associated with facial clefting, identify factors to predict outcomes in many areas including general health, wound healing, speech and psychological adjustment and how well do they do in education

at school and beyond. This will also inform future interventions to improve health outcomes for children born with clefts.

References

- Bearn D, Mildinhal S, Murphy T, Murray JJ, Sell D, Shaw WC, Williams AC, Sandy JR: Cleft lip and palate care in the United Kingdom – the Clinical Standards Advisory Group (CSAG) Study. Part 4: Outcome comparisons, training, and conclusions. *Cleft Palate Craniofac J* 2001;38:38–43.
- Concato J, Shah N, Horwitz RI: Randomized controlled trials, observational studies, and the hierarchy of research designs. *N Engl J Med* 2000;342:1887–1892.
- Mars M, Plint DA, Houston WJ, Bergland O, Semb G: The Goslon yardstick: a new system of assessing dental arch relationships in children with unilateral clefts of the lip and palate. *Cleft Palate J* 1987;24:314–322.
- Mars M, Asher-McDade C, Brattström V, Dahl E, McWilliam J, Mølsted K, Plint DA, Prah-Andersen B, Semb G, Shaw WC, The RPS: A six-center international study of treatment outcome in patients with clefts of the lip and palate. Part 3: Dental arch relationships. *Cleft Palate Craniofac J* 1992;29:405–408.
- Shaw WC, Dahl E, Asher-McDade C, Brattström V, Mars M, McWilliam J, Mølsted K, Plint DA, Prah-Andersen B, Roberts C, Semb G, The RPE: A six-center international study of treatment outcome in patients with clefts of the lip and palate. Part 5: General discussion and conclusions. *Cleft Palate Craniofac J* 1992;29:413–418.
- Attack NE, Hathorn IS, Semb G, Dowell T, Sandy JR: A new index for assessing surgical outcome in unilateral cleft lip and palate subjects aged five: reproducibility and validity. *Cleft Palate Craniofac J* 1997;34:242–246.
- Huddart AG, Bodenham RS: The evaluation of arch form and occlusion in unilateral cleft palate subjects. *Cleft Palate J* 1972;9:194–209.
- Pruzansky S, Aduss H: Arch form and the deciduous occlusion in complete unilateral clefts. *Cleft Palate J* 1964;30:411–418.
- Morris T, Roberts C, Shaw WC: Incisal overjet as an outcome measure in unilateral cleft lip and palate management. *Cleft Palate Craniofac J* 1994;31:142–145.
- Mølsted K, Asher-McDade C, Brattström V, Dahl E, Mars M, McWilliam J, Plint DA, Prah-Andersen B, Semb G, Shaw WC, The RPE: A six-center international study of treatment outcome in patients with clefts of the lip and palate. Part 2: Craniofacial form and soft tissue profile. *Cleft Palate Craniofac J* 1992;29:398–404.
- Williams AC, Bearn D, Mildinhal S, Murphy T, Sell D, Shaw WC, Murray JJ, Sandy JR: Cleft lip and palate care in the United Kingdom – the Clinical Standards Advisory Group (CSAG) Study. Part 2: Dentofacial outcomes and patient satisfaction. *Cleft Palate Craniofac J* 2001;38:24–29.
- Long RE, Hathaway R, Daskalogiannakis J, Mercado A, Russell K, Cohen M, Semb G, Shaw WC: The Americleft study: an inter-center study of treatment outcomes for patients with unilateral cleft lip and palate. Part 1: Principles and study design. *Cleft Palate Craniofac J* 2011;48:239–243.
- Hathaway R, Daskalogiannakis J, Mercado A, Russell K, Long RE, Cohen M, Semb G, Shaw WC: The Americleft study: an inter-center study of treatment outcomes for patients with unilateral cleft lip and palate. Part 2: Dental arch relationships. *Cleft Palate Craniofac J* 2011;48:244–251.
- Daskalogiannakis J, Mercado A, Russell K, Hathaway R, Dugas G, Long RE, Cohen M, Semb G, Shaw WC: The Americleft study: an inter-center study of treatment outcomes for patients with unilateral cleft lip and palate. Part 3: Analysis of craniofacial form. *Cleft Palate Craniofac J* 2011;48:252–258.
- Mercado A, Russell K, Hathaway R, Daskalogiannakis J, Sadek H, Long RE, Cohen M, Semb G, Shaw WC: The Americleft study: an inter-center study of treatment outcomes for patients with unilateral cleft lip and palate. Part 4: Nasolabial aesthetics. *Cleft Palate Craniofac J* 2011;48:259–264.
- Russell K, Long RE, Hathaway R, Daskalogiannakis J, Mercado A, Cohen M, Semb G, Shaw WC: The Americleft study: an inter-center study of treatment outcomes for patients with unilateral cleft lip and palate. Part 5: General discussion and conclusions. *Cleft Palate Craniofac J* 2011;48:265–270.
- Hathorn I, Roberts-Harry D, Mars M: The Goslon yardstick applied to a consecutive series of patients with unilateral clefts of the lip and palate. *Cleft Palate Craniofac J* 1996;33:494–496.
- Johnson N, Williams AC, Singer S, Southall P, Attack N, Sandy JR: Dentoalveolar relations in children born with a unilateral cleft lip and palate (UCLP) in Western Australia. *Cleft Palate Craniofac J* 2000;37:12–16.
- Morris DO, Roberts-Harry D, Mars M: Dental arch relationships in Yorkshire children with unilateral cleft lip and palate. *Cleft Palate Craniofac J* 2000;37:453–462.
- Zreagat M, Hassan R, Halim AS: Dentoalveolar relationships of Malay children with unilateral cleft lip and palate. *Cleft Palate Craniofac J* 2009;46:326–330.

- 21 Noverraz AE, Kuijpers-Jagtman AM, Mars M, van't Hof MA: Timing of hard palate closure and dental arch relationships in unilateral cleft lip and palate patients: a mixed-longitudinal study. *Cleft Palate Craniofac J* 1993;30:391–396.
- 22 Roberts CT, Semb G, Shaw WC: Strategies for the advancement of surgical methods in cleft lip and palate. *Cleft Palate Craniofac J* 1991;28:141–149.
- 23 Chawla O, Deacon SA, Attack NE, Ireland AJ, Sandy JR: The 5-year-olds' index: determining the optimal format for rating dental arch relationships in unilateral cleft lip and palate. *Eur J Orthod* 2011 (E-pub ahead of print).
- 24 Ozawa TO, Shaw WC, Katsaros C, Kuijpers-Jagtman AM, Hagberg C, Rønning E, Semb G: A new yardstick for rating dental arch relationship in patients with complete bilateral cleft lip and palate. *Cleft Palate Craniofac J* 2011;48:167–172.
- 25 Ross RB: Treatment variables affecting facial growth in complete unilateral cleft lip and palate. *Cleft Palate J* 1987;24:5–77.
- 26 Shaw WC, Asher-McDade C, Brattström V, Dahl E, McWilliam J, Mølsted K, Plint DA, Prah-Andersen B, Semb G, The RP: A six-center international study of treatment outcome in patients with clefts of the lip and palate. Part 1: Principles and study design. *Cleft Palate Craniofac J* 1992;29:393–397.
- 27 Semb G, Brattström V, Mølsted K, Prah-Andersen B, Shaw WC: The Eurocleft study: intercenter study of treatment outcome in patients with complete cleft lip and palate. Part 1: Introduction and treatment experience. *Cleft Palate Craniofac J* 2005;42:64–68.
- 28 Asher-McDade C, Brattström V, Dahl E, McWilliam J, Mølsted K, Plint DA, Prah-Andersen B, Semb G, Shaw WC, The RP: A six-center international study of treatment outcome in patients with clefts of the lip and palate. Part 4: Assessment of nasolabial appearance. *Cleft Palate Craniofac J* 1992;29:409–412.
- 29 Grunwell P, Brondsted K, Henningsson G, Janssonius K, Karling J, Meijer M, Ordling U, Wyatt R, Vermeij-Zieverink E, Sell D: A six-centre international study of the outcome of treatment in patients with clefts of the lip and palate: the results of a cross-linguistic investigation of cleft palate speech. *Scand J Plast Reconstr Surg Hand Surg* 2000;34:219–229.
- 30 Semb G, Brattström V, Mølsted K, Prah-Andersen B, Shaw WC: The Eurocleft study: intercenter study of treatment outcome in patients with complete cleft lip and palate. Part 1: Introduction and treatment experience. *Cleft Palate Craniofac J* 2005;42:64–68.
- 31 Brattström V, Mølsted K, Prah-Andersen B, Semb G, Shaw WC: The Eurocleft study: intercenter study of treatment outcome in patients with complete cleft lip and palate. Part 2: Craniofacial form and nasolabial appearance. *Cleft Palate Craniofac J* 2005;42:69–77.
- 32 Mølsted K, Brattström V, Prah-Andersen B, Shaw WC, Semb G: The Eurocleft study: intercenter study of treatment outcome in patients with complete cleft lip and palate. Part 3: Dental arch relationships. *Cleft Palate Craniofac J* 2005;42:78–82.
- 33 Semb G, Brattström V, Mølsted K, Prah-Andersen B, Zuurbier P, Rumsey N, Shaw WC: The Eurocleft study: intercenter study of treatment outcome in patients with complete cleft lip and palate. Part 4: Relationship among treatment outcome, patient/parent satisfaction, and the burden of care. *Cleft Palate Craniofac J* 2005;42:83–92.
- 34 Shaw WC, Brattström V, Mølsted K, Prah-Andersen B, Roberts CT, Semb G: The Eurocleft study: intercenter study of treatment outcome in patients with complete cleft lip and palate. Part 5: Discussion and conclusions. *Cleft Palate Craniofac J* 2005;42:93–98.
- 35 Clinical Standards Advisory Group: *Cleft Lip and/or Palate*. London HMSO, ISBN 0–11–322103–7.
- 36 Sell D, Grunwell P, Mildinhal S, Murphy T, Cornish TA, Bearn D, Shaw WC, Murray JJ, Williams AC, Sandy JR: Cleft lip and palate care in the United Kingdom – the Clinical Standards Advisory Group (CSAG) Study. Part 3: Speech outcomes. *Cleft Palate Craniofac J* 2001;38:30–37.
- 37 Sandy JR, Williams AC, Bearn D, Mildinhal S, Murphy T, Sell D, Murray JJ, Shaw WC: Cleft lip and palate care in the United Kingdom – the Clinical Standards Advisory Group (CSAG) Study. Part 1: Background and methodology. *Cleft Palate Craniofac J* 2001;38:20–23.
- 38 Williams AC, Sandy JR, Thomas S, Sell D, Sterne JA: Influence of surgeon's experience on speech outcome in cleft lip and palate. *Lancet* 1999;354:1697–1698.
- 39 Williams AC, Johnson NC, Singer S, Southall P, Mildinhal S, Semb G, Sell D, Thomas S, Sandy JR: Outcomes of cleft care in Western Australia: a pilot study. *Aust Dent J* 2001;46:32–36.
- 40 Clarkson J, Paterson P, Thorburn G, El-Ali K, Richard B, Hammond M, Wake M: Alveolar bone grafting: achieving the organisational standards determined by CSAG, a baseline audit at the Birmingham Children's Hospital. *Ann R Coll Surg Engl* 2005;87:461–465.
- 41 Revington PJ, McNamara C, Mukarram S, Perera E, Shah HV, Deacon SA: Alveolar bone grafting: results of a national outcome study. *Ann R Coll Surg Engl* 2010;92:643–646.
- 42 Felstead AM, Deacon S, Revington PJ: The outcome for secondary alveolar bone grafting in the South West UK Region Post-CSAG. *Cleft Palate Craniofac J* 2010;47:359–362.
- 43 Hathorn IS, Attack NE, Butcher G, Dickson J, Durning P, Hammond M, Knight H, Mitchell N, Nixon F, Shinn D, Sandy JR: Centralization of services: standard setting and outcomes. *Cleft Palate Craniofac J* 2006;43:401–405.
- 44 Tugwell P, Boers M, Brooks P, Simon L, Strand V, Idzerda L: OMERACT: an international initiative to improve outcome measurement in rheumatology. *Trials* 2007;8:38.

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Surgical Correction of Cleft Lip and Palate

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Abstract

Surgical cleft repair aims to restore function of the oro-nasal sphincter and oro-nasal soft tissues and re-establish the complex relationship between perioral and perinasal muscle rings without compromising subsequent mid-facial growth and development. Here we review the surgical anatomy of this region, optimal timing for surgical repair and current thinking on the use of surgical adjuncts. In addition, an overview of current surgical techniques available for the repair of cleft lip, cleft palate and velopharyngeal insufficiency is presented. Finally, we briefly discuss nasal revision surgery and the use of osteotomy, including distraction osteogenesis in the cleft patient.

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The main goals of surgical cleft repair are functional restoration of the oro-nasal sphincter and oro-nasal soft tissues, re-establishing the complex relationship between perioral and perinasal muscle rings and the unhindered promotion of mid-facial growth and development. A key basis for successful outcome is a clear understanding of the underlying surgical anatomy.

The lip and primary palate are formed by the successful fusion of medial and lateral nasal processes with the maxillary process at around the 5th week of gestation. If these fail to fuse, a cleft lip and alveolus deformity develops. Associated

impaired fusion of the secondary palate results in a cleft lip and palate deformity. The secondary palate, which represents the majority of the definitive hard and soft palate, is formed by rotation of the palatal shelves from a vertical position lateral to the tongue to a horizontal position cranial to the tongue, with subsequent mesenchymal fusion at around the 8th to 12th week of pregnancy. An isolated cleft palate deformity results from the incomplete fusion of these palatal shelves and hence cleft palate is a distinct entity from cleft lip and alveolus (cleft of the primary palate). In bilateral clefts of the lip, the prolabium does not contain any muscle tissue because this mesenchymal structure arises from the maxillary processes after fusion with the nasal processes during normal development.

The upper lip consists of a central vertical philtrum and the adjacent defining philtral columns. The orbicularis oris muscle is complex, composed of the pars marginalis and the pars peripheralis. The pars marginalis is the deep circumferential structure running between the two modiolus and provides for sphincter function and oral continence. The pars peripheralis runs in an oblique manner from the modiolus, interdigitates with the other perioral muscles and inserts into the dermis. It is this dermal insertion that leads to

the formation of the philtral pillars and the white roll of the lip. The peripheral fibres are primarily responsible for articulation and facial expression [1].

Cupid's bow is formed by the insertion of the levator labii superioris to the ipsilateral philtral pillars. In cleft lip, the white roll is absent with the underlying pars marginalis being hypoplastic and disoriented. In a bilateral cleft lip none of the above-mentioned structures are present in the absence of any muscle tissue in the prolabium. On the medial side of a unilateral cleft lip, there is a deficiency of vermillion width, whereas the width of the vermillion at the crest of the bow is normal or slightly increased in the lateral cleft segment. A lack of muscle tissue, narrow vermillion band and exposed hypoplastic mucosa characterise the prolabium in the bilateral complete cleft deformity [2].

There are three sets of interconnecting muscle rings between the infraorbital region to the chin, which form part of the fascial envelope in continuity with the superficial muscular aponeurotic system of the face. In patients with a cleft lip, the perinasal, perioral and perimental muscle rings are out of balance and abnormally attached. This leads to deviation of the facial axis towards the non-cleft side. The orbicularis oris attaches to the anterior nasal spine on the non-cleft side and alar base on the cleft side, which results in opposing forces splaying the cleft-sided alar cartilage, thus widening the nasal base. The alar cartilage is deformed but not hypoplastic, the lateral crus is lengthened and pulled laterally leading to foreshortening of the medial crus, flattening of the nasal dome and displacement of the alar base cranially on the affected side as a result of latero-cranial traction of the transverse part of the nasalis muscle (fig. 1). The premaxilla including the nasal septum is deviated towards the non-cleft side [3].

In unilateral cleft lip deformities the premaxilla, which is part of the greater cleft segment, is rotated outwards and the lesser cleft segment is collapsed medially and retro-positioned [4]. In complete bilateral clefts of the lip and alveolus, the



Fig. 1. Typical features of a unilateral cleft lip with splaying of the cleft-sided nostril and lateralisation of the premaxilla to the opposite side.

central segment, which is a remnant of the medial nasal processes, is composed of the prolabium and the premaxilla. Due to unrestrained cartilaginous growth and tongue pressure, the premaxilla is quite prominent and upward rotated. The nose becomes wide and flat with separation of the crural domes. Latham [5] studied the columella in bilateral clefts and found a normal cartilaginous skeleton in a deficient cutaneous envelope.

The soft palate contains five major muscle pairs: palatoglossus, palatopharyngeous, tensor and levator veli palatini and the musculus uvulae. The superior pharyngeal constrictor muscle forms the upper lateral and posterior pharyngeal wall and works in functional cooperation with the levator veli palatini during swallowing. The tensor veli palatini forms the palatal aponeurosis with its counterpart from the opposite side in the anterior third of the soft palate. It stretches the velum and helps open the eustachian tube. The levator veli palatini arises from the skull base and medial aspect of the eustachian tube and runs cranial to the tensor to fuse with its counterpart in the midline of the velum. This muscle pair forms a sling which lifts the soft palate in a cranio-posterior direction and plays the crucial role in the creation of the velopharyngeal seal, which is of paramount

importance for certain speech sounds. In a cleft patient the levator runs in a sagittal rather than transverse direction. It is attached to the aponeurosis of the tensor veli palatini. This malposition leads to the failure of the muscle sling and needs to be corrected in a cleft palate repair.

Delaire [6] and Joos [7] described the interaction of the primary and secondary growth centres in patients with cleft lip and palate. The primary growth centres are the chondral matrices of the skull base. The septo-vomerine centre plays the most important role in ventral growth. The secondary growth centres are the viscerocranial sutures where growth occurs by complex mechanisms of interaction between appositional growth of the adjacent bones and the intermediate periosteum and attached muscle fibres (musculoperiosteosutural apparatus). The facial primary and secondary growth centres are intimately connected and interact by virtue of the facial mimetic muscles and their attachments. The perinasal and perioral muscles attach to the antero-inferior part of the nasal septum. During ventro-caudal growth of the septo-vomerine growth centre, the muscles connecting this to the zygomaticomaxillary sutures exert dilating forces leading to the mid-facial rostral growth. Based on these findings, Delaire [6] postulated that growth retardation was a result of scarring from inappropriate surgical intervention. In patients with cleft lip and palate there is disruption of the complex interaction between the primary and secondary growth centres and only a functional repair of this link can achieve facial harmony [6–8].

Timing of Surgery

Many controversies still surround the optimal timing and sequence for the correction of cleft lip and palate deformity and no single universally accepted protocol exists. Historically the 'rule of 10s' was applied for the timing of cleft lip repair – an age of at least 10 weeks, with a weight of 10 lb

and haemoglobin level of 10 g/dl. Although these recommendations were made at a time of riskier paediatric anaesthesia, they are still appropriate. Advances in paediatric and neonatal anaesthesia have made earlier repairs possible when indicated. In most centres, cleft lip repair is carried out at 3–6 months of age. By this time, fetal physiology is replaced by that of an infant and the risks of anaesthesia are significantly reduced. The issue of timing of surgery for palatal repair has been even more controversial. There are varying schools of thought about the timing and staging of palatal repair and their implications on speech and facial growth and development. Early surgery has the potential advantage of better speech outcomes whereas delayed surgery is potentially associated with less impairment of mid-facial growth, but worse speech outcomes. The main aim of palate repair is restoration of soft palate function, especially with regard to normal speech development, as certain sounds need a build-up of oral air pressure, which relies on a compliant velopharyngeal seal. Hence to lay the foundations of normal speech, the repair should be carried out prior to the onset of speech, that is in the first year of life [9, 10].

Dorf and Curtain [11] looked at articulation abnormalities in early and delayed palatal closure, with 12 months used as an arbitrary dividing point between the two groups. They found a statistically significant difference between those groups, with a 10% incidence of pharyngeal and glottal articulation abnormalities in children who had repairs prior to 12 months of age and 86% incidence of articulation abnormalities in those who had had their repairs after 12 months of age. In 1996, Rorhich et al. [12] presented a study of 44 patients. The average age at follow-up was 17 years in the early and 18.2 years in the late repair groups. They noticed a higher rate of persistent fistulae (35%) in the late compared with 5% in the early repair group. This study also showed higher speech deficiencies with the delayed closure.

There is no obvious consensus on the staging of palatal repair. The main variants are (1) isolated

repair of lip first followed by simultaneous hard and soft palate repair a few months later and (2) simultaneous repair of lip and soft palate followed by a delayed closure of the hard palate (usually at about 18 months of age but possibly months or even years later). Other surgical sequences are followed much less frequently, such as lip and palate repair all-in-one procedure or palate repair first (hard and soft palate) followed by the lip repair a few months later. Rayner [13] concluded in 1925, from his series of 125 cases, that there was greater dental arch collapse if the hard palate was repaired in the first 2 years of life than if the repair was carried out at 3 or 4 years. A two-stage repair was popularized by Schweckendiek [14] in the 1940s. He began closing the soft palate early followed by delayed repair of the hard palate in late childhood to achieve normal speech and better maxillary growth. His methods were analysed by Bardach et al. [15], who found good facial growth but a high incidence of velopharyngeal insufficiency and articulation problems. Koberg and Koblin [16] showed in their large series of 2,000 patients that early surgery before 1 year did not cause greater maxillary growth inhibition compared to surgery at other ages. The maximal growth inhibition resulted from palatal surgery during the second maxillary growth spurt at 8–15 years. The authors further compared facial growth relationships in over 1,000 patients and noted greatest growth hindrance from push-back procedures followed by the von Langenback relaxing incision method. They felt that the timing of the cleft palate repair was not the major deterrent to facial growth but the surgery itself may cause the growth retardation. The authors concluded that the avoidance of palatal surgery at 8–12 years of age during the rapid maxillary growth phase is advisable.

A one-stage all-in-one cleft lip and palate repair was first published by Farina in 1958 for patients with unilateral cleft lip and palate. De Mey et al. [17] published in 2009 their non-randomized prospective study comparing the results of the all-in-one technique to a non-cleft control group and a staged closure protocol (lip and palate closed

separately). Their results suggested that both the operative groups had similar maxillofacial growth outcomes. Although this paper presents outcomes at 10 and 15 years of age, the authors recommended evaluation of craniofacial growth after completion of skeletal development. The all-in-one procedure does have the benefits of one single anaesthesia and reduced burden of care for parents.

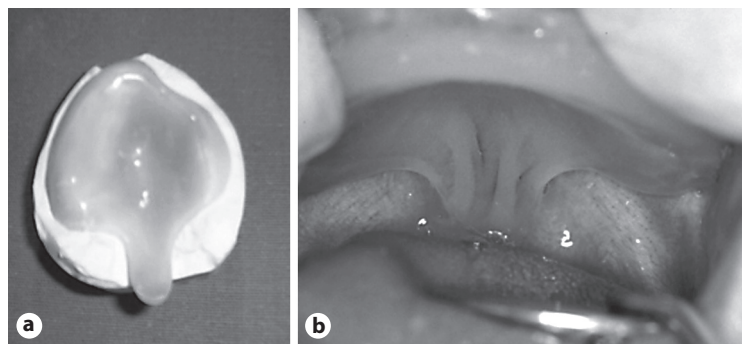
Adjuncts to Surgery

A number of adjunctive techniques have been described, which aim to improve surgical outcome. These include pre-surgical orthopaedics, naso-alveolar moulding, lip adhesion and gingivoperiosteoplasty.

Pre-Surgical Orthopaedics

The main aim of pre-surgical orthopaedics is realignment of the malpositioned alveolar processes and premaxilla prior to surgery. This leads to a more anatomically correct alignment of both the hard and the attached soft tissues of the cleft, facilitating a tension-free surgical repair. The use of pre-surgical orthopaedic appliance therapy is still quite controversial. Their application varies widely, with some surgeons using it for most cases, some occasionally, others only in cases of bilateral clefts and some do not use it at all. Several appliance designs have been described over the years. Broadly speaking, pre-surgical orthopaedics can be classified as extra- and intra-oral appliance therapy. Extra-oral devices apply forces to the maxillary arches indirectly through the labial soft tissues and can simply be surgical tape applied across the upper lip or elasticated straps (strapping). Intra-oral devices in turn can be active or passive, as well as self-retaining or bone-anchored. In most cases these appliances are cheap, simple in their design and easy to apply but need parental cooperation for maintenance (fig. 2). They are most useful in bilateral cleft cases with a protruding and upward rotated premaxilla. Intra-oral appliances have also been described that help

Fig. 2. Hotz plate on plaster model (a) and in situ (b) with a soft palate extension for muscle stimulation.



to align the cleft segments via growth guidance through regular adjustments [18, 19].

After the lip repair they prevent the interposition of the tongue into the palatal cleft and avoid widening of the defect through tongue pressure. Bone-anchored appliances, such as the Latham co-axial screw device, provide active forces to mould the maxillary processes [20]. The device is placed at 6 weeks and after 2–3 months the appliance can be removed simultaneously with a preliminary lip adhesion and gingivo-periosteoplasty in preparation for definitive lip repair a few months later. There are studies which suggest that this procedure can have a negative effect on mid-facial growth.

Naso-Alveolar Moulding

Naso-alveolar moulding (NAM) is a technique of early stenting and reshaping of the cleft nose deformity. This adds on to the above-described concept of pre-surgical orthopaedic alignment of the maxillary arch. The technique was first described by Grayson et al. [20] in 1993 and was based on research for moulding cartilage by Matsuo et al. [21]. They believed that the high oestrogen levels at the time of birth correlates with raised hyaluronic acid levels which in turn inhibit the cross-linking of cartilaginous matrix. NAM utilises this malleability by the progressive moulding of immature nasal cartilaginous skeletal components. The benefits of NAM are improvement

of nasal tip projection, alar base width reduction and columellar lengthening. Nasal stents are utilised and attached to the palatal orthopaedic plate either directly or by wires. Taping of the prolabium can also be combined for columella and prolabium lengthening if indicated. The patients need frequent follow-up for close monitoring and regular adjustments. The most common complication of this procedure is irritation of skin and nasal mucosa. Special care needs to be taken to avoid necrosis of nasal tissue due to excessive pressure.

Lip Adhesion

Preliminary lip adhesion to transform a complete cleft into an incomplete one was popularised by Randall [22] in the 1960s. Lip adhesion is mainly used in difficult complete clefts like wide unilateral and bilateral, and clefts with misaligned maxillary segments. Sceptics of surgical lip adhesions argue that the produced scarring interferes with the subsequent definitive lip repair and the alignment of the cleft segments could equally be achieved by plates and strapping.

Gingivo-Periosteoplasty

Gingivo-periosteoplasty was first described by Skoog [23] in 1965, using flaps to create a periosteal tube in the cleft alveolus, which acts as a matrix to induce bone growth in the defect. Millard and Latham [24] reported a modification to position

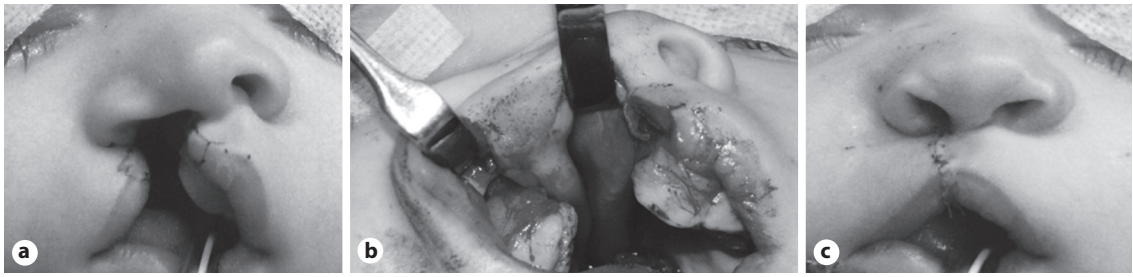


Fig. 3. Delaire technique: (a) incision design, (b) wide soft tissue mobilisation for tension free repair, and (c) end of surgery. Note the small triangle above the vermilion border to lengthen the lip and break the straight-line scar.

the alveolar segments, dissection of the mucoperiosteum out of the cleft and creation of a mucoperiosteal tunnel across the bony gap, setting up a conducive environment for bone formation and eventual tooth eruption in the cleft area. Santiago and Grayson's group [25] reported that 60% of patients in their series who underwent pre-surgical alveolar moulding and gingivo-periosteoplasty at the time of lip repair did not need any subsequent alveolar bone grafting.

Cleft Lip Repair

The first reported repair of cleft lip was performed by an unknown physician during the Chin dynasty in 390 AD [4]. Early methods of cleft lip repair were straight-line closure techniques as described by Rose [26] in 1891 and then Thompson [27] in 1912. In the 1940s and 1950s, techniques based on triangular flap modification techniques were proposed by Blair and Brown [28], and Brown and McDowell [29]. Le Mesurier's quadrilateral flap technique [30] and Tennison-Randall's triangular flap technique [31] both introduced tissue into the lower part of the lip and produced an aesthetically pleasing cupid's bow and pouting of the tubercle. In 1955, Millard [32] introduced the principle of downward rotation of the medial segment and the lateral flap advancement into the upper lip. Millard's original rotation advancement

procedure preserves cupid's bow and philtral dimple, however it destroys the philtral line in the subnasal area and distributes the tension of the repair at the alar base, possibly leading to a better moulding of the underlying alveolus. This technique was not originally aiming at a functional repair of the peri-oral and peri-nasal muscle slings. Subsequently, several modifications were described aiming to lengthen the columella and decrease alar base scarring. In the 1970s, Delaire [6] emphasised the importance of a functional muscle repair (fig. 3). His procedure respects the anatomical boundaries between the lip and the nose and avoids scars crossing the alar rim and columellar base. Wide sub-periosteal undermining of the cleft-sided anterior maxilla to release the peri-oral muscles, careful muscle dissection and anatomical repositioning of not only the peri-oral but also the peri-nasal muscles contribute towards achieving lip length and aesthetics with this technique. Delaire re-introduced a straight-line incision but combined it with a functional muscle repair, which counteracts potential lip shortening by scar contraction. The introduction of a small supra-vermillion triangle (fig. 3) (much smaller than in the Tennison-Randall procedure) leads to a nice rounding of cupid's bow at its highest point as the scar runs parallel to the vermilion border in this area [33].

Management of bilateral cleft lip has changed over time. Modern repair techniques utilise the

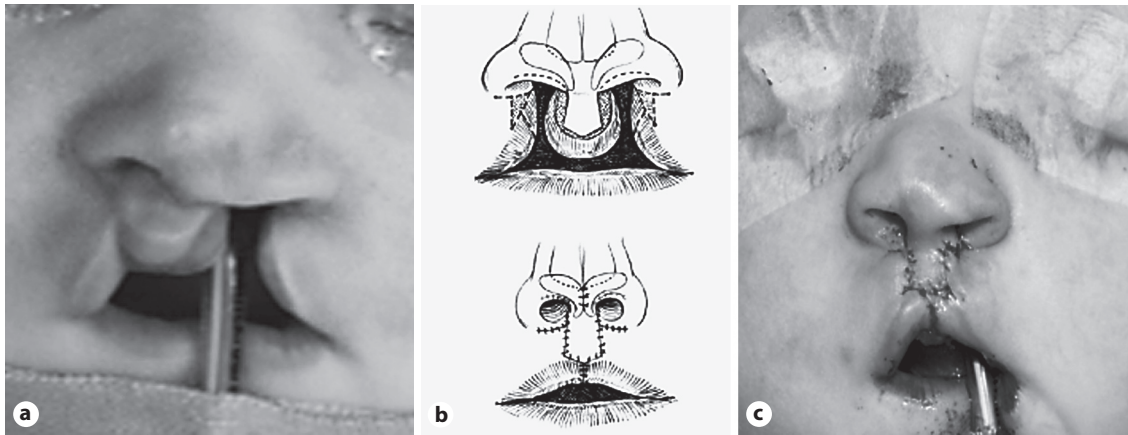


Fig. 4. **a** Patient with a bilateral cleft lip (incomplete at right and complete at the left-hand side). **b** Flap design by Mulliken. **c** Post-surgical repair, note the vermilion is formed entirely from the lateral lip segments.

prolabium to form the mid-portion of the upper lip (fig. 4). In 2000, Mulliken [34] described the principles of surgical reconstruction of the bilateral cleft lip. According to him these are: maintenance of symmetry, securing primary muscular union, correct design of the prolabial flap, formation of the median tubercle and the vermilion-cutaneous ridge by tissue from the lateral segments. Several types of bilateral lip repair are in use but the scope of this article does not permit detailed description of individual procedures. In general, a straight-line closure technique is useful except in the case of a very small prolabium, where Millard's techniques can be preferable. Some authors have described a staged sequential closure of wide bilateral cleft lips side by side. In contrast to simultaneous bilateral closures, this approach can increase difficulty in achieving lip symmetry.

Cleft Palate Repair

In 1862, Bernhard von Langenbeck [35] suggested palatal closure with two bipediced muco-periosteal palatal flaps mobilised medially. This technique is in principle still in use today. Ernst

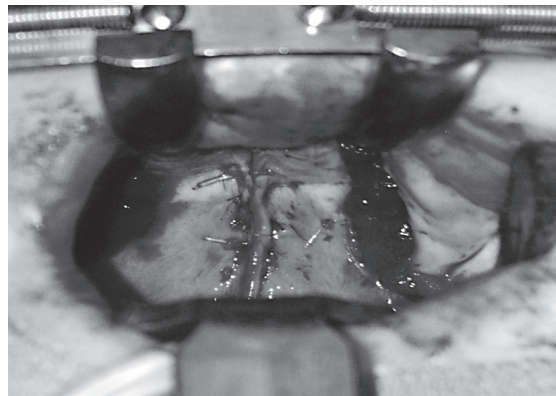


Fig. 5. Cleft palate repair with bilateral bipediced flaps and lateral releasing incisions for healing by free granulation.

published in 1925 a modification, introducing some sort of muscle repair, division of the palatine vessels and formation of parapharyngeal pouches for reduction of tension on the midline sutures (fig. 5). A three-layer closure of the soft palate, dissection of the nasal mucosa and posterior based unipediced flaps for velum lengthening by V-Y closure were developed by Victor Veau in the 1930s. Kilner [36] and Wardill [37]

developed a similar 'push-back' procedure in the 1920s and early 1930s. This technique has the disadvantages of leaving denuded areas on the palate antero-laterally, with associated scar contraction, growth impairment and fistula formation. There is also uncertainty about long-term stability of the gained palatal length. The detachment of ill-attached velar musculature from the posterior aspect of the palatine bone and its anatomically correct repair was first emphasised by Kriens [38] in 1967. This important concept of intra-velar veloplasty is still widely used and a basis of modern primary and secondary cleft palate repair. The technique of a double opposing Z-plasty for simultaneous velar closure and lengthening was introduced by Furlow [39] in 1978 who published the method in 1986. This technique has the advantage of reorienting and retro-positioning the levator muscles more anatomically with less muscle dissection, thus minimizing scarring around the muscles. Lengthening of the velum and narrowing of the naso-pharyngeal orifice are also useful outcomes of this method, which has gained popularity and is widely used in many centres around the world.

Correction of Velopharyngeal Incompetence

A competent velopharyngeal seal mechanism is essential for normal speech. An inability to occlude the velopharyngeal aperture leads to velopharyngeal insufficiency or incompetence (VPI). There have been various surgical procedures described for the correction of VPI in patients who have had previous surgery for repair of their cleft palate. Intra-velar veloplasty with or without palatal lengthening, Furlow's palatoplasty, sphincter or velopharyngeal flap pharyngoplasty as well as augmentation of the posterior pharyngeal wall are the most common methods applied for correction of VPI. Alloplastic augmentation of the posterior pharyngeal wall has gone out of favour due to the associated infection risk, extrusion and migration

of the implants. Secondary Furlow's palatoplasty and intra-velar veloplasty are in modern VPI surgery the most physiological procedures and hence have enormously gained popularity. The details of the individual methods are outside the scope of this chapter.

Primary Management of the Cleft Lip Nose

Correction of the cleft nose deformity is one of the most difficult and controversial aspects of the surgical management of these patients. It presents to varying extents in most cleft lip cases. These deformities have been described as three-dimensional problems involving all tissue layers of the nose. As already mentioned, cleft nose deformities have characteristic features and vary with the extent of clefting and whether a unilateral or bilateral cleft lip is present. The main goals of primary cleft nose surgery are the closure of the nasal floor and sill as well as repositioning of the alar base and the lower lateral (alar) cartilages. There has been some reluctance in the past to operate on the nose prior to completion of facial growth, to avoid disturbance of growth centres in the developing nose. However, there is lack of evidence to show that repositioning of lower lateral cartilages and suture refinement of the tip leads to growth interference. McComb and Coghlan [40] showed in their 18 years' longitudinal study no significant growth disturbance in children who underwent primary cleft rhinoplasty and the symmetry gained during the surgery was maintained into adult life. Primary cleft rhinoplasty is performed commonly in the unilateral cleft deformity where asymmetry is almost always present. This procedure can be accomplished through the standard lip repair incisions and involves the mobilization of the alar base from the piriform rim and separation of the alar base attachments. The floor of the nose is closed at the correct level. The lower lateral cartilages are dissected from the overlying skin and positioned appropriately. Nasal tip correction can

be achieved with internal sutures or tie-over bolsters, resulting in better projection, symmetry and tip definition. Conformers may be used to achieve a more pleasing shape of the nostril.

Correction of Facial Skeletal Deformity

Hypoplastic mid-facial development is common in cleft lip and palate and can result from a combination of congenital maxillary growth reduction as well as an effect of scar tissue following primary or secondary surgical repair of the cleft palate. Classically this presents as a combination of a narrow and retruded maxilla with a class III skeletal pattern. Traditionally, orthognathic surgery with Le Fort I osteotomy with or without concurrent mandibular procedures have been used for correction. Since the 1990s distraction osteogenesis of the facial skeleton has become a viable alternative for the management of such deformity. Both methodologies aim to advance the retruded maxilla to improve the severely deformed facial profile, which can be one of the most disturbing stigmata of cleft patients. The problems anticipated with the maxillary osteotomy and immediate advancement in CLP patients are associated with the pre-existing scarring, less predictable vascularity, the degree of advancement and post-surgical skeletal relapse. Maxillary distraction is carried out with the use of either intra-oral or extra-oral appliances, whilst palatal distractors are used where transverse dimension of the maxilla needs to be improved. A meta-analysis published by Cheung and Chua [41] in 2006 looked at the evidence base of cleft osteotomy and distraction. This study concluded that distraction was preferred to orthognathic surgery in younger patients with more marked mid-facial deficiency. Intra-operative complications were uncommon with both techniques, although this might have been an underestimation as most studies reviewed were retrospective in nature. The known complications of conventional

surgery are intra-operative haemorrhage, avascular necrosis or rarely avulsion of the osteotomised segment and oro-nasal communication. Distraction can be associated with misdirected vectors, device failure, skin irritation, a painful distraction process and in very rare cases even blindness. There is a lack of conclusive data in the meta-analysis on the differences between surgical relapse, velopharyngeal function and speech between the two techniques. Both these procedures provide an improvement in facial aesthetics and correction of the bite.

Future Outlook

Cleft surgery has evolved over the years due to a better understanding of the concepts of facial growth and aetiopathogenesis of cleft lip and palate. Evolution of surgical techniques, inclusion of newer technologies like distraction osteogenesis, use of microsurgical repair techniques and improved three-dimensional imaging modalities have helped to achieve better results. The development of integrated multidisciplinary teams has not only streamlined care provision but also aims to improve the experience of the patient and their families. The major obstacle in further evidence-based development of cleft surgery remains the timescale between initial procedure and final outcome. The true result of an applied method in primary cleft surgery can only be fully judged after the individual patient has finished growing at about the age of 17–20 years. An individual surgeon will only complete treatment from start to finish in a small number of patients.

The science of cleft surgery can be further improved by sharing knowledge between various centres globally and setting up multi-centre longitudinal randomised control trials to evaluate the efficacy of the currently applied techniques and treatment schedules.

References

- Nicolau PJ: The orbicularis muscle: a functional approach to its repair in the cleft lip. *Br J Plast Surg* 1983;36:141.
- Mulliken JB, Pensler JM, Kozakewich HP: The anatomy of Cupid's bow in normal and cleft lip. *Plast Reconstr Surg* 1993; 92:395.
- Talmant JC: Nasal malformations associated with unilateral cleft lip. Accurate diagnosis and management. *Scand J Plast Reconstr Surg Hand Surg* 1993;27:183.
- Burt JD, Byrd SJ: Cleft lip: unilateral primary deformities. *Plast Reconstr Surg* 2000;105:1043.
- Latham RA: Development and structure of the premaxillary deformity in bilateral cleft lip and palate. *Br J Plast Surg* 1973; 26:1.
- Delaire J: Theoretical principles and techniques of functional closure of the lip and nasal aperture. *J Maxillofac Surg* 1978;6:109.
- Joos U: The importance of muscular reconstruction in the treatment of cleft lip and palate. *Scand J Plast Reconstr Surg* 1987;23:109.
- Anastassov GE, Joos U: Comprehensive management of cleft lip and palate deformities. *J Oral Maxillofac Surg* 2001;59: 1062.
- Huppa CT: Primary repair of cleft palate; in Langdon J, Patel M, Ord R, Brenan P (eds): *Operative Oral and Maxillofacial Surgery*. London, Hodder Arnold, 2011, pp 509–607.
- Rohrich R, Love EJ, Byrd S, Johns DF: Optimal timing of cleft palate closure. *Plast Reconstr Surg* 2000;106:413–421.
- Dorf D, Curtin JW: Early cleft palate repair and speech outcome. *Plast Reconstr Surg* 1982;70:74–81.
- Rohrich RJ, Roswell AR, Johns DF, et al: Timing of hard palate closure: a critical long-term analysis. *Plast Reconstr Surg* 1996;98:236.
- Rayner HH: The operative treatment of cleft palate: a record of results in 125 consecutive cases. *Lancet* 1925;i:816.
- Schweckendiek H: Zur Frage der Früh- und Spätoperationen der angeborenen Lippen-Kiefer-Gaumenspalten (in German). *Z Laryngol Rhinol Otol* 1951;30:51.
- Bardach J, Morris HL, Onlin WH: Late results of primary veloplasty: the Marburg project. *Plast Reconstr Surg* 1984; 73:207.
- Koberg W, Koblin J: Speech development and maxillary growth in relationship to technique and timing of palatoplasty. *J Maxillofac Surg* 1973;1:44.
- De Mey A, Franck D, Cuyllits N, et al: Early one stage repair of complete unilateral cleft lip and palate. *J Craniofac Surg* 2009;20:1723.
- Hotz M, Gnoinski W: Comprehensive care of the cleft lip and palate children at Zurich University: a preliminary report. *Am J Orthod* 1976;70:481.
- Latham RA: Orthodontic advancement of the cleft maxillary segment: a preliminary report. *Cleft Palate J* 1980;17:227.
- Grayson BH, Cutting C, Wood R: Preoperative columella lengthening in bilateral cleft-lip and palate. *Plast Reconstr Surg* 1993;92:1422.
- Matsuo K, Hirose T, Ottagiri T, Norose N: Repair of cleft lip with nonsurgical correction of nasal deformity in the early neonatal period. *Plast Reconstr Surg* 1989;83:25.
- Randall P: A lip adhesion operation in cleft surgery. *Plast Reconstr Surg* 1965;35:371–376.
- Skoog T: The use of periosteal flaps in the repair of clefts of the primary palate. *Cleft Palate J* 1965;2:332.
- Millard DR Jr, Latham RA: Improved primary surgical and dental treatment of clefts. *Plast Reconstr Surg* 1990;86:856–871.
- Santiago P, Grayson CB, Cutting MP, et al: Reduced need for alveolar bone grafting by presurgical orthopaedics and primary gingivoperiosteostomy. *Cleft Palate Craniofac J* 1998;35:77.
- Rose W: *Hare Lip and Cleft Palate*. London, H.K. Lewis, 1891.
- Thompson JE: An artistic and mathematically accurate method of repairing the defect in cases of harelip. *Surg Gynaecol Obstet* 1912;14:498.
- Blair VP, Brown JB: Mirault operation for single hare lips. *Surg Gynaecol Obstet* 1930;51:81.
- Brown JB, McDowell F: Simplified design for repair of single cleft lip. *Surg Gynecol Obstet* 1945;80:12.
- LeMesurier AB: Method of cutting and suturing lip in complete unilateral cleft lip. *Plast Reconstr Surg* 1949;4:1.
- Tennison CW: The repair of unilateral cleft lip by the stencil method. *Plast Reconstr Surg* 1952;9:115.
- Millard DR Jr: A primary camouflage of the unilateral harelook; in Skoog T (ed): *Transactions of the First International Congress of Plastic Surgery*. Baltimore, Williams & Wilkins, 1957, pp 160–166.
- Markus AF, Delaire J: Functional primary closure of cleft lip. *Br J Oral Maxillofac Surg* 1993;31:281–291.
- Mulliken JB: Primary repair of bilateral cleft lip and nasal deformity. *Plast Reconstr Surg* 2000;108:181–194.
- Von Langenbeck B: Die Uranoplastik mittels Ablösung des mucosperiostalen Gaumenüberzugs (in German). *Arch Klin Chir* 1862;2:25.
- Kilner TP: Cleft lip and palate repair techniques. *St Thomas Hosp Rep* 1937;2:127.
- Wardill WEM: Cleft palate. *Br J Surg* 1928;16:127–148.
- Kriens OB: An anatomical approach in veloplasty. *Plast Reconstr Surg* 1969; 43:29.
- Furlow L: Cleft palate repair by double opposing Z-plasty. *Plast Reconstr Surg* 1986;78:724–735.
- McComb HK, Coghlan BA: Primary repair of the unilateral cleft lip nose: completion of a longitudinal study. *Cleft Palate Craniofac J* 1996;33:23–31.
- Cheung LK, Chua HDP: A meta-analysis of cleft maxillary osteotomy and distraction osteogenesis. *Int J Oral Maxillofac Surg* 2006;35:14–24.

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Orthodontic Treatment in the Management of Cleft Lip and Palate

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Abstract

The orthodontic treatment of patients with all types of cleft lip and palate, a resume of facial growth and discussion on dental and occlusal development is presented. A fully integrated cleft team provides life-long interdisciplinary holistic treatment for patients born with an orofacial cleft. To understand the team approach to cleft care, this article should be read in close conjunction with those on speech therapy, surgery and alveolar bone grafting to determine the synergy required between these and other clinical specialties. Team working is essential to produce successful patient outcomes. Cleft teams and their constituent clinicians are at the forefront of patient outcome assessment and any aspiring cleft team member must understand how the continuous evaluation of outcome and burden of care will further refine clinical protocols for future patients.

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The orthodontist's role in the management of patients with orofacial clefting is challenging, rewarding and as varied as the different types of cleft encountered. No two clefts are the same and each patient has different needs, aspirations and desires. There are several occasions during a child's first two decades of life where orthodontic intervention may be required. These packages of orthodontic care are often designed to

optimise the jaws and dentition for the intervention of other specialities, particularly the surgical, restorative, dental and speech therapy teams. Quality orthodontic care hinges upon the prerequisite of excellent oral hygiene and a pristine or well-restored, disease-free dentition. Active disease (fig. 1) must be treated urgently and completely and a preventative regimen instigated. There is evidence that children with an intraoral cleft have high dental caries experience [1] and to counter this, at all stages, an aggressive preventative regime should be encouraged. Regular liaison with the general dental practitioner and paediatric dental specialist, as well as the oral hygienist, is crucial to enable the orthodontist to deliver a quality outcome. The cleft specialist orthodontist will offer care at different developmental stages and these treatments may last many months or even several years. These prolonged phases of contact with the patient and their family places the orthodontist in the position of the patient's advocate. The orthodontist often facilitates onward referral to other specialists, both dental and medical, within and beyond the cleft team. The supporting roles of patient groups such as CLAPA (Cleft Lip and Palate Association) in the UK are also invaluable. Centralised services in the UK have also seen



Fig. 1. Untreated active dental disease in a patient with a complete bilateral cleft lip and palate.

an increase in the return of adult patients within their third decade and beyond. This patient cohort is returning for advice and treatment to maintain, restore and replace failing treatments undertaken in childhood. Adult patients who refused treatments as children and adolescents also return seeking advice in later life.

All members of the cleft team are acutely aware that the burden of care for patients with orofacial clefting must be kept to a minimum at all times. Centralised services may increase the distances patients have to travel to access their care and therefore time away from social and educational commitments can be significant. It is important that these interruptions to a child and their family are kept to a minimum. Orthodontic treatment episodes can be particularly lengthy and it is important that clear, reasonable and achievable objectives are set out at the beginning of treatment. The cleft specialist orthodontist will endeavour to ensure that these episodes are as concise as reasonably practicable. This will aid patients to remain committed and focused on the end result, maintaining an optimum level of oral health throughout.

It is incumbent upon all cleft teams to prospectively and longitudinally evaluate the outcome of all the interventions undertaken. In this way and by contributing to multicentre research, care pathways and treatment protocols may be streamlined for the benefit of future patients. The orthodontist is key in collecting a number of different records that demonstrate clinical outcomes for the patient born with orofacial clefting. UK cleft teams are obliged to collect records as described by the Craniofacial Society of Great Britain and Ireland (<http://www.craniofacialsociety.org.uk/info/audit.html>). These and other outcome measures are discussed elsewhere.

Presurgical Maxillary Orthopaedics

Presurgical maxillary orthopaedics (PSO) aims to realign the bony sections of the upper jaw and reduce the cleft space, excluding the tongue from the cleft, improving the relations of the cleft segments prior to initial surgical correction, and is not a new concept [2]. Retaining orthodontic appliances on cleft edentulous infant alveolar ridges is not without complication. Some appliances are fixed directly to the alveolar segments with pins, wires [3] or screws, but these techniques should now be abandoned due to the inevitable collateral damage to developing tooth germs [4]. Intraoral acrylic mouth plates used with elastic lip tapes or straps made from medical dressings and plasters have a much lower morbidity and may be utilised to prepare the patient with orofacial clefting for surgery. There is no subject more controversial in the treatment of the child with orofacial clefting than the efficacy of PSO. Popularised by McNeil [5] in the 1950s, many different versions, modifications and applications have been described. Intraoral mouth plates may be utilised in conjunction with lip tapes or straps which can be applied to the child's face or a head cap. Plates may be passive or active, completely intraoral or with extraoral components added to improve

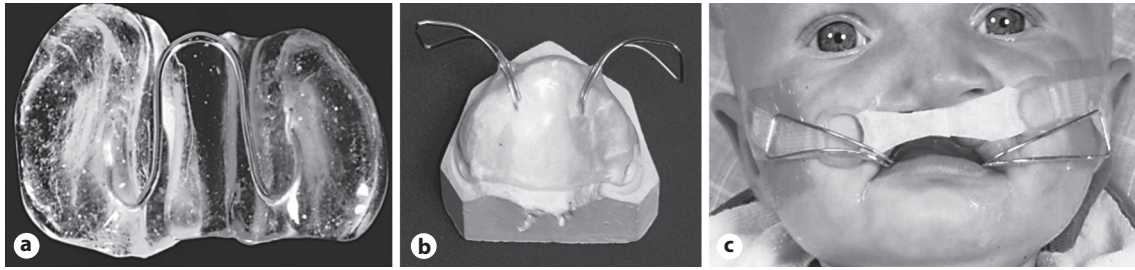


Fig. 2. PSO. **a** DiBiase-type active appliance. **b** Modified passive presurgical orthopaedic intraoral plate with extraoral wings to aid retention. **c** Appliance in situ with facial strapping to apply traction.

retention. Extraoral projections are added to intraoral plates in the nasoalveolar moulding technique, which is designed to modify columellar length and nasoalar form [6].

Cleft teams who favour the use of PSO have a tailored surgical protocol where surgical timings suit their overall treatment philosophy. Centres may employ a delayed surgical protocol where lip repair is completed at 12 months and palate repair at 18 months rather than the 6 and 12 months widely adopted in the UK. Teams may also choose to repair the alveolar cleft 'early', when the segments are appropriately approximated. Some graft the alveolus with autologous bone, whilst others repair the periosteum across the cleft without grafting. This gingivoperiosteoplasty technique [7] relies upon the repaired periosteum and associated haematoma from this and the surrounding tissues within the cleft alveolus to heal, organise and generate bone in situ.

There are two main types of active intraoral appliances or mouth plates (fig. 2). The first is constructed by pouring up plaster models from intraoral impressions. Over the weeks and months prior to the primary surgical correction, sequential impressions are taken. The model is cut, sectioned and repositioned little by little to make the desired movements of the maxillary cleft segments. Acrylic plates are made to fit the adjusted model and sequentially fitted in the mouth. The second type of active intraoral plate was popularised by

DiBiase and Hunter [8] and is again made from an intraoral model. A wire spring incorporated between two halves of the appliance permits manipulation of the cleft segments and usually this single appliance can be worn up until the primary surgery and occasionally beyond. The two sections of the appliance overlap one another and in so doing, the oral and nasal cavities remain separated, even if the appliance is expanded. This continuity excludes the tongue and also prevents milk and fluids passing between the mouth and the nasal cavity, preventing trauma to the delicate intranasal tissues, particularly those covering the nasal septum.

Passive plates are also constructed from plaster models made from intraoral impressions. Movements of the cleft segments are planned and carried out in several different ways. By adding plaster to alter the cleft dimensions on the model, the shape of the alveolar processes can be manipulated in the laboratory. Once the plate is worn intraorally, manipulation of the cleft segments of the maxilla may be encouraged. Alternatively, Hotz's technique [9] avoids the application of any lip tape or strapping and employs sequential adjustments to the acrylic base plate directly, rather than manipulating the model. In this approach, the patient is recalled every few weeks and the plate adjusted by selective removal of acrylic from the fitting surface of the appliance, to mould the segments as desired. One may add acrylic to the base

plate on the lateral aspects of the alveolar ridges and remove it from the cleft areas so as to encourage growth into the space between segments with the aim, as per all techniques, of reducing the cleft dimensions.

During development at around 6 weeks in utero, disruption of the dental lamina may cause variation in the number, shape, size of the teeth and can negatively influence the enamel quality of the teeth adjacent to the cleft. Neonatal or natal teeth may first erupt in the early weeks immediately post-partum and will likely take up a position in the mouth around the alveolar cleft (fig. 3). These tend to be a rudimentary shell of enamel of no functional or aesthetic benefit. Neonatal teeth may traumatise the mother when breast-feeding and may also interfere with treatment when PSO is employed. The usual treatment for such an early and functionless tooth is extraction. These teeth also pose a risk to the infant's airway and if evident at the time of lip repair they should be extracted then. They may be removed at any time by securing them in surgical gauze and extracting under digital pressure.

Any attempt to adjust the form of the cleft prior to surgery is intensive, both from a clinical and laboratory production perspective and the technique adds significantly to the burden of care. Is the weight of evidence strongly supportive of the technique of PSO? Not so. The technique is employed by teams for all cleft types, and there is little doubt that, when skilfully applied, the technique does reduce the size of an intraoral maxillary arch cleft. Some surgeons do report that the reduced inter-cleft segment dimensions makes surgical correction simpler. There is, however, no convincing evidence to support this hypothesis. Despite this however, there is much evidence that does not support any long-term benefit of the use of PSO for cleft lip and palate patients. A Cochrane Systematic Review [10] found no evidence PSO aids feeding and improves growth and weight for height of the cleft infant. Papers



Fig. 3. Right-sided neonatal tooth in an infant with a complete bilateral cleft lip and palate

reporting on the inter-centre Dutchcleft studies concur and report no improvement in maxillary arch dimensions [11] following the use of PSO. There is also no improvement in anteroposterior maxillo-mandibular relations when PSO has been employed in infancy.

PSO remains a controversial subject within cleft lip and palate care. It is regrettable that both patients and providers and funders of clinical care cannot with certainty determine which treatment choices are best for an individual. With contributions from centralised, high-volume teams, the weight of evidence will point us towards determining the ideal protocol. Clinicians will continue to face challenges where the light of science is not focused enough to equivocally point the way. It is therefore of paramount importance for all medical and surgical patients that the art of clinical practice is not forgotten. The clinician who has had a specialist training and is able to evaluate contemporary clinical and scientific evidence will likely adopt a pragmatic approach to clinical problems. Those able to evaluate new evidence and techniques as they come to light and combine those with long established treatments will offer much to our patients.

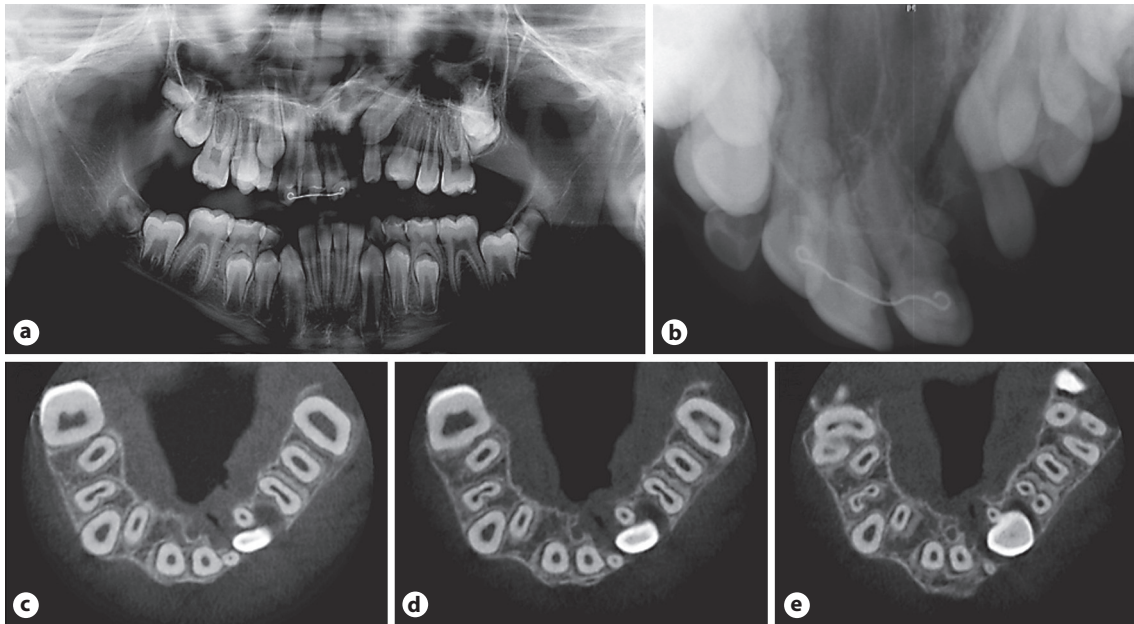


Fig. 4. Panoramic (a) and upper standard occlusal (b) radiographs of a child with a complete unilateral cleft lip and palate demonstrating a late developing, unerupted supernumerary tooth adjacent to the UL1, transposition of the UL23 and potential molar stacking in the left maxilla. Cone-beam CT images (c–e) confirm the supernumerary's position and the degree of transposition, as well as the size and dimension of the cleft alveolus.

Early Orthodontics (5–8 Years Old)

The first major multidisciplinary outcome audit point for a child born with an orofacial cleft in the UK occurs in the year following their fifth birthday. As the transition into the permanent dentition begins, renewed vigour should be given to the dental disease-preventative regime. Consideration should be given to fissure-sealing permanent molars. Periapical infection affecting the primary dentition adjacent to the alveolar cleft should be aggressively treated prior to bone grafting.

The dental development of a child with an orofacial cleft should be closely monitored as the embryological disturbance that produced the cleft will likely also have disrupted the development of the deciduous and permanent dentition and the chronology of eruption [12]. The teeth

affected may not be immediately adjacent to the cleft but commonly the number, position, shape, size, enamel quality and colour may be affected [13]. Supernumerary teeth may be multiple and the lateral incisor may be missing but where present may be found on either side of the cleft, on the lesser or greater segment. The precise complement of teeth present adjacent to the cleft may first be identified on the pre-alveolar bone graft radiograph films but, especially where supernumerary teeth are present, three-dimensional imaging by cone-beam CT scanning can be of help to identify the precise position of the teeth and associated structures (fig. 4). Maxillary arch length is commonly reduced and this may manifest itself when the first molar teeth begin to erupt. These teeth may become impacted beneath the second deciduous molars [14] and may cause the child pain and discomfort. Extraction of the deciduous

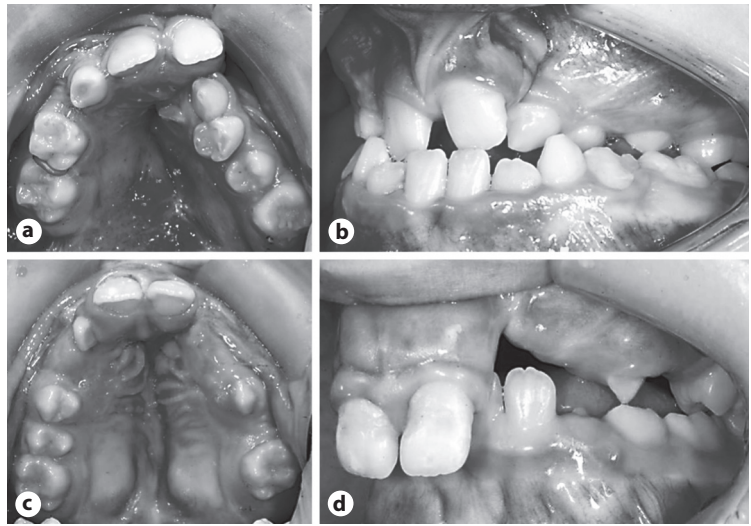


Fig. 5. Typical dental and occlusal features in complete unilateral (left-sided) cleft lip and palate (**a, b**) and bilateral cleft lip and palate (**c, d**).

molar may be indicated but careful consideration of the likely future orthodontic space requirement needs to be given.

Unilateral cleft maxillary alveolar segments are often contracted with anterior and unilateral posterior crossbites present (fig. 5). The incisor relationship is commonly class III and the lesser maxillary cleft segment is tipped medially. There is reduced overbite or cleft-centred open bite and incisor teeth are missing, tipped into the cleft, ectopic, impacted and rotated. There are also commonly maxillary centreline problems. The bilateral cleft patient, on average, presents with bilateral posterior crossbites, increased overjet and overbite with a prominent, often slightly mobile premaxilla carrying the maxillary incisors in an anterior position, often retroclined with reasonably arranged midline relations (fig. 5).

A class III incisor relationship may develop regardless of the maxillo-mandibular skeletal base relationships. If incisal and skeletal relations are favourable, interceptive orthodontic treatment may be appropriate. The use of simple removable appliances, sectional fixed orthodontic appliances or perhaps full-arch, stopped wires may be

employed to move single or multiple upper incisors 'over the bite'. Care must be taken where alveolar clefting exists, as it is important not to move permanent teeth into the cleft and compromise dental vitality.

The cleft specialist orthodontist may be called upon for assistance to augment the care offered by the speech and language therapy team. Oral impressions for the manufacture of an electropalatography appliance may be indicated. Electropalatography is a technique producing a visual aid for patients having speech therapy when tongue placement needs to be improved. Multiple electrodes are embedded in the acrylic base plate of an upper removable appliance and these are connected to a computer, which produces a live stream of tongue to palate contact images on a computer screen. The therapist and patient are then able to work together with this visual feedback to reinforce the therapy. Occasionally, where speech sounds have a nasal quality, a non-surgical approach to combat velopharyngeal insufficiency may be required. Orthodontic appliances that obturate palatal fistulae and palatal tissue deficits or appliances that elevate a short or poorly lifting

soft palate may be manufactured. Palatal function assessment may require speech recordings, video x-ray and endoscopic examination. During the construction and tailoring of a palatal lift or obturator appliance, the same assessments are employed to ensure accurate manufacture, adaption and modification of the appliance and maximising its clinical efficiency.

The relationship of teeth, jaws and oral soft tissues may influence the production of certain speech sounds. Skeletal base relationships and hence the relationship of the teeth to the tongue can affect speech production. On occasion, bilateral and labiodental sounds may be difficult to reproduce. Palatalization and lateralization of alveolar speech sounds is commonly encountered [15]. The degree to which a patient is affected may be related to the severity of the skeletal discrepancy. This may improve once relationships are normalised after orthognathic surgery and speech therapy is often a crucial adjunct to help the patient overcome their incorrect but well-established cleft-type speech patterns. The patient who has persistent speech concerns may often benefit from the addition of tissue volume into the base of the soft palate. Tissue may be surgically rotated and turned back distally from the buccal mucosa into the palatal tissues. A vascular pedicle may persist long after the buccal flap tissues have fully healed and collaterally vascularised in situ. This pedicle may be traumatised, generating symptoms of pain and discomfort, as new permanent molars erupt. The potential timing depends upon the eruption schedule for first or second molars and their relative position to the pedicle as it crosses the occlusal table. The buccal flap, now fully integrated, is no longer dependent upon the pedicle and trauma to it may spontaneously resolve but on occasion may require surgical division. The orthodontist may also be called upon in advance of buccal flap surgery to prevent a newly created pedicle being traumatised and potentially compromising vitality. In this situation, the occlusion may require temporary 'opening' ideally with a fixed

bite plane, until the tissues have healed perhaps 4–6 weeks post-operatively.

Post-Alveolar Bone Graft Development and Orthodontics (8–12 Years Old)

After alveolar bone grafting, the cleft specialist orthodontist will pay close attention to oral hygiene, local healing, the subsequent dental development and the developing and changing skeletal jaw relations of the child with an orofacial cleft.

In the right conditions, with excellent oral hygiene, in the immediate post-alveolar bone graft phase, the bone surgically augmented to the alveolar cleft will reorganise and mature. The cleft specialist orthodontist will have stabilised the teeth and the alveolar gap prior to the repair, to fix the maxillary segments in situ. In unilateral cleft alveolar cases a transpalatal arch alone will usually suffice. Occasionally a fixed appliance may be employed to prepare and retain the unilateral cleft but more commonly, a bilateral cleft will require more rigid and direct stabilisation in this way (fig. 6) or with a cemented occlusal coverage fixed rigid splint. During this phase of alveolar bone graft maturation, the first post-operative outcome radiographs will be taken, but not until the cleft tooth is erupted so that the final outcome will be known and the success of the bone graft be understood in relation to predetermined outcome measures [16]. It is of interest that a bone graft may be clinically deemed to be a success when radiographic evidence is to some degree to the contrary.

It may be months or years following the alveolar bone graft before the teeth immediately adjacent to the cleft erupt. The lateral incisor is commonly palatally displaced from the dental arch and it is reported that the canine adjacent to the cleft may fail to erupt in up to 20% of cases [17]. Radiographic evaluation, often with films for parallax or cone-beam CT, may be required to assess whether or not surgical exposure and a closed eruption technique is of benefit. Rarely, additional

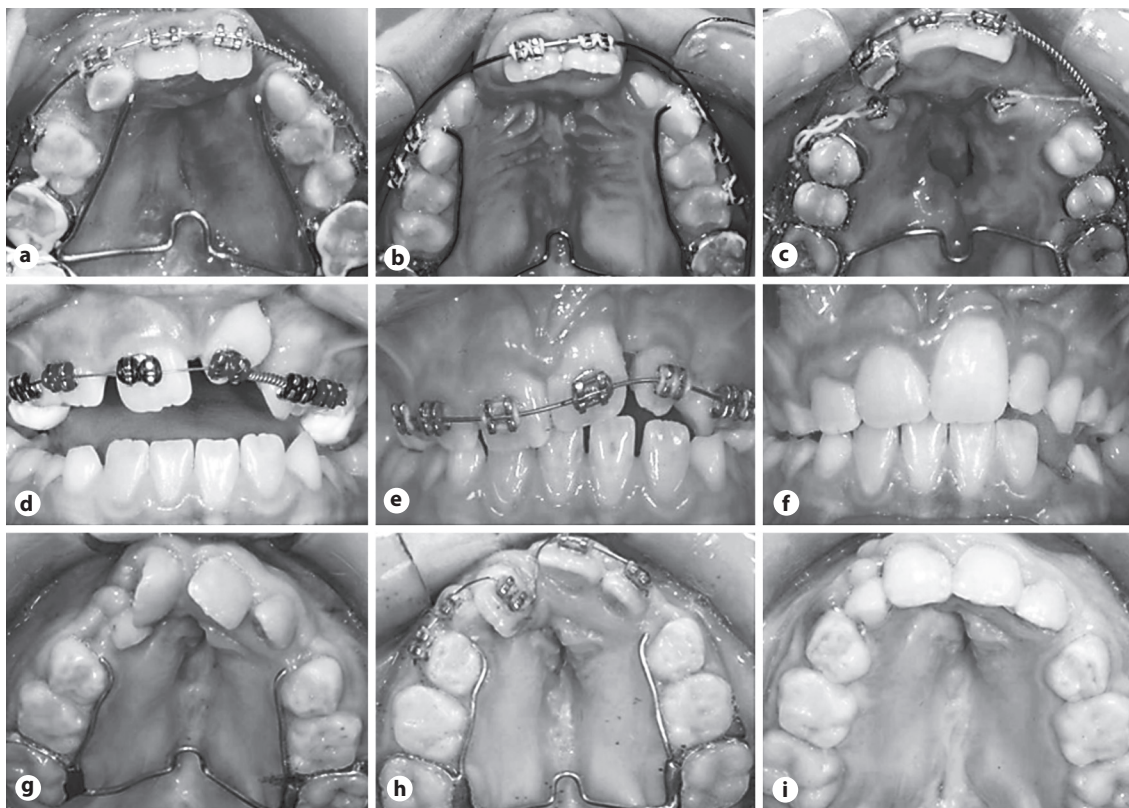


Fig. 6. Orthodontic treatment at the time of alveolar bone grafting. Complete left-sided cleft lip and palate (**a**) and bilateral cleft lip and palate stabilised with transpalatal arches prior to alveolar bone grafting (**b**). Orthodontic traction applied to bilateral ectopic and impacted maxillary canine teeth (**c**). Bond position on teeth adjacent to bone graft needs consideration prior to and post alveolar bone graft (**d-f**). Correction of rotated incisor teeth with fixed appliance post alveolar bone graft (**g-i**).

grafting may be required but this should be confined to cases where no gold chain is employed, as this may act to transport bacterial infection into the surgical site. Traction to impacted teeth will result in an extended treatment time and may well envelop the definitive orthodontic treatment phase as the patient moves into the full permanent dentition (fig. 6).

In the phase immediately after alveolar bone grafting, children in the UK are often in their final years of primary school education, preparing for life at secondary school. This time often coincides with the desires of increasingly self-aware

children to fit in with their peers and be of 'average appearance'. It is of benefit to those children with clefts if interceptive treatments are completed prior to this school change. Orthodontic fixed appliance brackets placed on the teeth adjacent to the alveolar cleft prior to grafting should deliberately be positioned so as not to move tooth apices into the cleft. Once surgery has been successfully completed, brackets will be repositioned to allow the teeth to be fully as aligned and uprighted as possible (fig. 6). Often, minor occlusal and positional inaccuracies of the teeth may be accepted as this is often an interceptive phase of

treatment with limited objectives. The patient and clinician are aware that a further phase of treatment may follow and complete correction undertaken. Once the success of the alveolar bone graft has been assured, the correction of rotated, tipped and angulated incisors may be undertaken, often with fixed orthodontic appliances (fig. 6). In general, appliance systems with high values of maxillary arch incisor buccal crown torque and lingual crown torque in the mandibular arch are of value where compensation is required to overcome lesser class III skeletal bases. On completion of this phase of orthodontic care, the child is often still in the mixed dentition and the use of removable retention is unreliable. The employment of temporary bonded retainers is advisable and discrepancies between gingival heights will improve as the tissues mature.

In the years following alveolar bone grafting, the skeletal jaw relations of the child with an orofacial cleft will change. On average, the young child with a surgically treated complete unilateral cleft lip and palate has bimaxillary retrusion, relative protrusion and asymmetry in the premaxilla, with increased maxillary arch width. It is of interest that the mandible is often described as being retrognathic. There is also decreased posterior vertical maxillary height and nasal septal cartilages are commonly deviated towards the cleft [18]. The Sri Lankan studies of individuals with unoperated cleft lip and palate [19] demonstrates the natural history and growth of individuals who have not had the opportunity of any corrective surgery. This gives observers the unique opportunity to analyse the natural history of the maxillo-mandibular complex in individuals with an orofacial cleft when growth is not affected by the surgical procedures that offer valuable and life-long benefit. The average Sri Lankan individual with an unoperated complete unilateral cleft lip and palate has a well-positioned maxilla but with significant protrusion of the upper labial segment and increased overjet, prominence of the major cleft segment and mild contraction

of the minor segment but rarely to the extent of buccal crossbite. Maxillary width is generally reduced and a retrusive mandible seen, results similar to those seen in the reported Norwegian growth studies [20].

Commonly, class III skeletal bases are encountered in the cleft population and the experienced orthodontic clinician will make a clinical assessment of the degree of skeletal discrepancy before determining if incisor relations and crossbites should be corrected. It is an obvious desire to see skeletal bases that have grown well to class I proportions, but class II relationships may also be encountered in the cleft population. Children with Pierre Robin sequence [21] or bilateral cleft lip and palate [22] may present with class II skeletal bases and these may lend themselves to growth modification treatment with conventional functional appliances. Class III tendencies are most commonly encountered where maxillary growth has been less than ideal and these are of higher incidence in the cleft population. Where maxillary growth has been severely reduced and facial appearance is of concern to the child, early/interceptive maxillary distraction osteogenesis may be considered. The temptation for prolonged, unreliable treatments involving a maxillary protraction facemask should be resisted, unless as part of the retention regime after distraction osteogenesis.

Conventional Orthodontics (11–15 Years Old)

Having accepted the skeletal base position, adopting a treatment plan that supports or that creates a class I incisor relationship is crucial. Extraction in the lower arch to reduce crowding and retrocline the incisors will likely be supported by a plan to match arch widths, keeping the maxillary incisors forward but permitting centreline correction in the unilateral cleft case. Increased overbite can be an issue in the bilateral case. Where a frank class III skeletal base relationship exists, maxillary

dental arch crowding alone can be treated. Class III skeletal bases can be ignored but care taken to ensure that the occlusion is not compromised for future decompensation in advance of later orthognathic treatment.

The next most significant question is what to do with the teeth, or lack of teeth, around the cleft alveolus. The lateral incisor is commonly missing or so severely ectopic as to require extraction. Consideration to extracting the contralateral lateral incisor tooth or a premolar will be given, especially where centreline issues exist. When the lateral incisor is missing, a non-extraction approach is likely to be taken in the maxilla on the cleft side in the unilateral case. Where the lateral incisor is to be substituted by the canine, modification of the shape of the canine can be undertaken and the lateral space closed. Rarely, this space may be maintained or possibly opened up for restoration with bridgework or implants. This can have the beneficial effect of increasing maxillary arch length and helping maintain a class I incisor relationship but commits the patient to life-long restorative maintenance. Gingival heights may need adjustment but this should be assessed when they are deemed to be mature. Where present, the diminutive lateral incisor may be moved into the arch and its dimensions improved with the addition of composite material. Cleft specialist orthodontists may deliberately keep the upper incisors proclined and in an anterior position, encouraging the creation of increased overjet so as to give additional support for the upper lip.

Orthodontic retention for the patient with a bony alveolar cleft needs to be permanent and reinforced with both removable and fixed techniques. This is the case for patients following comprehensive orthodontics as well as those who undergo a combined approach involving either orthognathic or distraction surgery. Special considerations need to be given to retention where restorative work has been placed. Maxillary arch width reduction after treatment will often have effects both on the buccal and notably the incisal

relations. Negative overjet may return after late mandibular growth or relapse of proclined incisors perhaps caused by a tight upper lip and associated scar tissue. Where arch width reduces, overbite may also reduce to the extent that open bite occurs around the bony alveolar cleft.

Orthognathic and Osteogenic Distraction Surgical Orthodontics

Where the ratio of mandibular growth exceeds that of the maxilla, a class III facial and dental relationship is likely to be seen. Orthodontic preparation for the patient requiring orthognathic surgery who has a repaired alveolar cleft must take into account the previous surgical history and an evaluation of the interventions to date. Full static, functional, radiographic and clinical evaluation of the presenting patient must be undertaken. A cephalometric evaluation should be recorded and assessment made. Digital two- and three-dimensional photographic and radiographic imaging should be undertaken, supported by video, study models, recorded speech samples and psychological evaluation. Where earlier treatments have produced suboptimal results, adjunctive retreatment should be undertaken prior to jaw surgery. Consideration should also be given to the extraction of third molar teeth. To identify patients at risk from a deterioration of their soft palate function, the effect of maxillary advancement on the velopharynx must be assessed prior to embarking upon a plan to alter the position of the maxilla. Speech assessment and video fluoroscopy are essential to fully consent a patient whose speech is at risk of becoming more nasal and this forms the basis for prediction as to whether further surgical correction of the palate may be possible [23].

Where the skeletal base relationship is beyond the scope of correction by orthodontic means alone, surgical repositioning of the maxilla and/or mandible should be considered (fig. 7). Ideally the maxilla should be moved the full extent of the discrepancy but a compromise of the final facial



Fig. 7. Bimaxillary osteotomy with a large maxillary movement to correct a significant class III malocclusion in a cleft patient. The post-surgical position has been maintained with rigid internal fixation using titanium miniplates.

form may be considered where it is felt that speech will be negatively affected by the full maxillary advancement. In this case the mandible may be set back too but ultimately the patient should be fully counselled and involved in the decision-making process leading up to the agreement of a final and comprehensive plan. Orthodontic treatment should be undertaken to decompensate the arches where appropriate. Extraction in the mandibular arch should generally be avoided to allow maxillary advancement as much as is functionally and

aesthetically desired. A rigid fixed orthodontic appliance with a prescription to encourage mandibular incisor proclination and maxillary incisor retroclination is advantageous. Tip and torque should be corrected and the arches should be levelled, aligned and coordinated. Anterior open bite should be increased prior to surgery and the planned final inter-cuspalation maximised by altering tooth widths with inter-proximal enamel reduction. Where segmental surgery is indicated, the segments should be individually levelled and

space created for the surgical cuts. Bracket positions or bracket type should be adapted to facilitate these cuts by moving the roots of the adjacent teeth out of the surgical field.

Where surgical movements required to correct a class III facial form are in excess of a 10-mm maxillary movement, consideration should be given to osteogenic distraction. This may either be undertaken with internal or external devices and the orthodontic preparation for patients requiring either of these approaches is broadly as for orthognathic surgery. The distraction appliances should be fixed directly to the craniofacial skeleton and not via the teeth and/or to orthodontic appliances. After maxillary/mandibular osteotomy, the judicious use of seating and guiding elastics should be facilitated by a rigid fixed appliance to help to produce the final and optimal facial and occlusal outcome.

Late and Retreatment Adult Orthodontics

Adult patients often seek opinions regarding possible options for new treatments and also advice regarding revision of procedures carried out in

their childhood as well as maintenance of work done years earlier. Some patients require advice only or minor interventions to achieve an optimal result whilst others require comprehensive retreatment. If retreatment is planned, this will likely commit an adult patient to a demanding and protracted treatment plan. Serious consideration should be given both to the burden of care and the clinical risk to the tissues of a now mature individual. Careful consideration of retreatment needs to be given both by the patient and by the clinical team and a thorough risk-benefit analysis undertaken prior to embarking on comprehensive care. Although an ideal outcome is what all want to see, a compromise may be required where disease processes have affected the orofacial tissues or when full commitment to the plan is not practical. As much time as is reasonably practical needs to be allowed for a patient to make their mind up before embarking on a multidisciplinary treatment plan. Consultation with the team psychologist and the use of simulations as well as meetings and discussions with other patients will all add benefit to the decision-making process.

References

- 1 Ahluwalia M, Brailsford S, Tarelli E, Gilbert S, Clark D, Barnard K, Beighton D: Dental caries, oral hygiene, oral clearance in children with craniofacial disorders. *J Dent Res* 2004;83:175–179.
- 2 Winters J, Hurwitz D: Presurgical orthopedics in the surgical management of unilateral cleft lip and palate. *Plast Reconstr Surg* 1995;95:755–764.
- 3 Brophy T: Cleft lip and palate. *J Am Dent Assoc* 1927;14:1108–1115.
- 4 Latham R: Orthopaedic advancement of the cleft maxillary segment: a preliminary report. *Cleft Pal J* 1980;17:227–233.
- 5 McNeil C: Orthodontics in the treatment of cleft palate. *Dent Rec* 1950;70:126–132.
- 6 Grayson B, Cutting C, Wood R: Preoperative columella lengthening in bilateral cleft lip and palate. *Plast Reconstr Surg* 1993;92:1422–1423.
- 7 Millard D: *Principles of Plastic Surgery*. Boston, Little, Brown, 1986.
- 8 DiBiase D, Hunter S: A method of presurgical oral orthopaedics. *Br J Orthod* 1983;10:25–31.
- 9 Hotz M: Pre- and early post-operative growth guidance in cleft lip and palate cases by maxillary orthopaedics. *Cleft Pal J* 1969;6:361–372.
- 10 Bessell A, Hooper L, Shaw W, Reilly S, Reid J, Glennly A-M: Feeding interventions for growth and development in infants with cleft lip, cleft palate or cleft lip and palate. *Cochrane Database Syst Rev* 2011;2:CD003315.
- 11 Prah C, Kuijpers-Jagtman A, Van't Hof M, Prah-Andersen B: A randomized prospective clinical trial of the effect of infant orthopedics in unilateral cleft lip and palate: prevention of collapse of the alveolar segments (Dutchcleft). *Cleft Pal Craniofac J* 2003;40:337–342.
- 12 Duque C, Dalben Gda S, Aranha A, Carrara C, Gomide M, Costa B: Chronology of deciduous teeth eruption in children with cleft lip and palate. *Cleft Pal Craniofac J* 2004;41:285–289.
- 13 Ranta R: A review of tooth formation in children with cleft lip/palate. *Am J Orthod Dentofac Orthoped* 1986;90:11–18.

- 14 Bjerklín K, Kurol J, Paulín G: Ectopic eruption of the maxillary first permanent molars in children with cleft lip and/or palate. *Eur J Orthod* 1993;15:535–540.
- 15 Albery E, Grunwell P: Consonant articulation in different types of cleft lip and palate; in Grunwell P (ed): *Analysing Cleft Palate Speech*. London, Whurr, 1993, pp 83–111.
- 16 Berglund O, Semb G, Abyholm F: Elimination of the residual alveolar cleft by secondary bone grafting and subsequent orthodontic treatment. *Cleft Pal J* 1986; 23:175–205.
- 17 Van der Wal K, van der Meulen B: Eruption of canines through alveolar bone grafts in cleft lip and palate. *Ned Tijdschr Tandheelkd* 2001;108:401–403.
- 18 Hermann N, Jensen B, Dahl E, Bolund S, Kreiborg S: A comparison of the craniofacial morphology in 2-month-old unoperated infants with unilateral complete cleft lip and palate, and unilateral incomplete cleft lip. *J Craniofac Genet Dev Biol* 1999;19:80–93.
- 19 Mars M, Houston WBA: A preliminary study of facial growth and morphology in unoperated male unilateral cleft lip and palate subjects over 13 years of age. *Cleft Pal J* 1990;27:7–10.
- 20 Semb G: A study of facial growth in patients with unilateral cleft lip and palate treated by the Oslo CLP Team. *Cleft Pal Craniofac J* 1991;28:1–21.
- 21 Hermann N, Kreiborg S, Darvann T, Jensen B, Dahl E, Bolund S: Early craniofacial morphology and growth in children with Robin Sequence. *Cleft Pal Craniofac J* 2003;40:131–43.
- 22 Hermann N, Darvann T, Jensen L, Dahl E, Bolund S, Kreiborg S: Early craniofacial morphology and growth in children with bilateral complete cleft lip and palate. *Cleft Pal Craniofac J* 2004;41:424–438.
- 23 Sell D, Harding A, Grunwell P: GOS.SPASS'98: an assessment for speech disorders associated with cleft palate and/or velopharyngeal dysfunction (revised). *Int J Lang Commun Disord* 1999;34:17–33.

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Alveolar Bone Grafting

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Abstract

In the 1970s, Boyne and Sands published reports on a new technique for alveolar bone grafting. They recommended that only cancellous bone be used and that the procedure be undertaken in the mixed dentition prior to canine eruption. Alveolar bone grafting prior to canine eruption soon became a routine part of the protocol for 90% of European and North American cleft teams. Several uncertainties remain however, such as the specifics of the surgical and orthodontic procedures, type of bone and donor site, and the best way to manage the space in the dental arch. Probably the commonest timing of the bone graft falls between 8 and 11 years, however there has been a trend in some centres to graft earlier in the hope of better outcome for the unerupted incisors. The influence on maxillary growth of earlier grafting has not been ascertained. A wide range of donor sites has been used but iliac crest remains the most popular. Many teams perform orthodontics prior to grafting to correct severe segment displacement or align incisors to improve surgical access. Following grafting, absence of the lateral incisor may be managed with orthodontic space closure, placement of an implant or bridgework. The introduction of alveolar bone grafting probably represents one of the most significant clinical innovations in cleft care. Hopefully, advances in tissue engineering will replace the need for transplantation of autogenous bone, or will provide an in-situ biological solution to the generation of a continuous bone fill across the alveolar cleft.

Prior to the introduction of alveolar bone grafting, closure of the cleft was associated with several limitations for patients with complete cleft lip and palate [1–3]. Many patients had residual oronasal fistulae despite several attempts to close them with soft tissue surgery. In the cleft region, nasal reflux through fistulae and/or food impaction in mucosal recesses caused chronic periodontal inflammation and eventual loss of teeth, despite good oral hygiene [3]. The bony defect in the alveolus limited orthodontic treatment, and all patients needed prosthetic restoration in the cleft region. In patients with bilateral clefts the mobility of the premaxilla made the retention of bridgework difficult. The need for prosthetic restorations had several disadvantages, for in addition to the general undesirability of artificial teeth for long-term aesthetics and dental health [3–6] lack of investing bone often precluded the correction of anterior tooth irregularities.

The first attempts at autogenous bone grafting to restore the alveolar cleft were made at the beginning of the last century [7, 8]. According to Koberg [9] the modern era of bone grafting in patients with clefts was introduced by Axhausen [10] whose ideas implied a re-establishment of the tooth-bearing function of the cleft site. Shortly after Axhausen's publication, reports on primary bone grafting (usually as a split rib graft) in early childhood appeared [10–12] and this was adopted in several countries. However, some years after

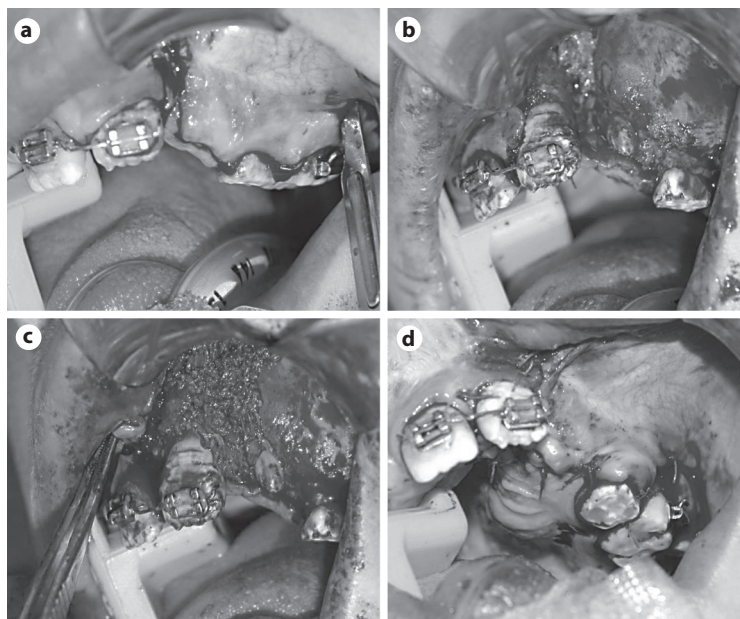


Fig. 1. Alveolar bone grafting: (a) incision lines, (b) the bony cleft widely exposed, (c) bone chips from the iliac crest are packed into the bony defect, and (d) careful suturing. Courtesy of Dr. Michael Matzen, Oslo University Hospital, Rikshospitalet, Norway.

the introduction of primary bone grafting, several studies reported serious impairment of subsequent maxillary growth [13–16] and bone grafting came to be viewed with great suspicion and was abandoned at most centres.

In 1965, Skoog [17] introduced periosteoplasty as an alternative to bone grafting: by using double-layer periosteal flaps, bone formation across the cleft was induced without the need of a donor site. The periosteoplasty technique was adopted by a few teams and in the 1990s the technique was modified and popularized by Brusati and Mannucci [18] and Cutting and Grayson [19] as gingivoalveoloplasty. However, concerns about reliability and subsequent growth persist.

Bone Grafting in the Mixed Dentition

In the 1970s, Boyne and Sands [20, 21] published reports on a new technique for alveolar bone grafting (fig. 1). They recommended that

only cancellous bone be used and that the procedure be undertaken in the mixed dentition prior to canine eruption. They maintained that osteogenic cells would survive in a fresh autograft and heal rapidly. This was confirmed by Albrektson [22] who detected the first vessels in cancellous bone 5–8 days after grafting and by 21 days observed that the graft was fully vascularised and exhibited osteogenesis. Boyne and Sands [21] initially presented results of 10 patients who had received this treatment and reported that with close collaboration between surgeon and orthodontist, a completely normal interdental septum could be achieved and the canine could erupt or be orthodontically moved into the new bone. Thus, a continuous dental arch without the use of prosthetic restoration, and improved periodontal conditions was achieved. This technique seemed to have great potential and several subsequent reports from other centres confirmed good outcome [23–28]. Alveolar bone grafting prior to canine eruption thus became a routine part

of the protocol for 90% of European and North American cleft teams [29–30]. Several uncertainties remain however, such as the specifics of the surgical and orthodontic procedures, type of bone and donor site, and the best way to manage the space in the dental arch.

Terminology and Goals of Bone Grafting

Turvey et al. [31] have suggested that the term ‘alveolar’ grafting is misleading as the nasal floor and lateral piriform rim are also constructed during the procedure performed today. The term ‘secondary’ bone grafting is certainly misleading as it is the first attempt to repair the defect in the alveolus [32]. However, the term is well established in everyday practice. Accordingly, the following terminology has been proposed: (1) primary bone grafting: bone grafting in the first 2 years of life; (2) early bone grafting: between 4 and 7 years; (3) mixed dentition bone grafting: between 7 and 12 years, and (4) late bone grafting: bone grafting after the eruption of the permanent dentition. The goals of contemporary alveolar bone grafting are primarily to eliminate the bony defect, making orthodontic space closure or implant placement possible; provide bony support for the adjacent teeth to ensure long-term periodontal health; close oronasal fistulae; eliminate mucosal recesses, thus making oral hygiene easier; provide support to the alar base, and stabilize the premaxilla in patients with complete bilateral cleft lip and palate.

Timing of Bone Graft

When Boyne and Sands reported their experience of bone grafting in the mixed dentition in 1972 and 1976 [20, 21], some were cautious about its adoption, given the adverse facial growth observed following primary bone grafting. As the sagittal and transverse growth of the anterior maxilla continues until the age of 8–9 years [33],

many considered it appropriate to postpone grafting till 8–11 years, and subsequent reports confirmed that this timing did not impair further facial growth [26, 34]. Some proposed that bone grafting could be done even earlier to provide bone for the erupting central incisor and the lateral incisor (when present) [35–38]. It was argued that this may avoid the gingival recession sometimes observed on the cleft side central incisor and allow the lateral incisors, if present, a better chance of root development. This timing and subsequent growth have yet to be formally investigated. Indeed, a broader range of timings is now common, grafting at around 7 years or earlier for those with a useful lateral incisor (about 28–37% of laterals [25, 38]) to just before canine eruption where retention of a lateral is not intended or the lateral is congenitally missing (fig. 2). It is suggested that delaying the graft in these circumstances may reduce canine impaction [39].

Pre- and Post-Grafting Orthodontics

It is generally considered useful to undertake some orthodontic fixed appliance preparation prior to grafting [40]. Where the cleft side central incisor has tipped into the cleft site and made surgical access to the bony defect difficult, this can be corrected with 4–5 months with fixed appliances [41]. In patients with severe displacement of the lateral segment, rotation of the lateral segment(s) outwards will facilitate placement of the graft. Using a quad helix or any other fixed appliance, segment repositioning is done within 6–8 months [41]. Segment reorientation cannot be obtained after grafting [42] and the expansion is also thought to reduce the frequency of canine impaction. Stabilizing a mobile premaxilla with orthodontic arch wire is common in patients with complete bilateral cleft lip and palate. Typically the arch wires will be removed during surgery, and replaced at the end of the operation to provide retention for around 3 months [40].

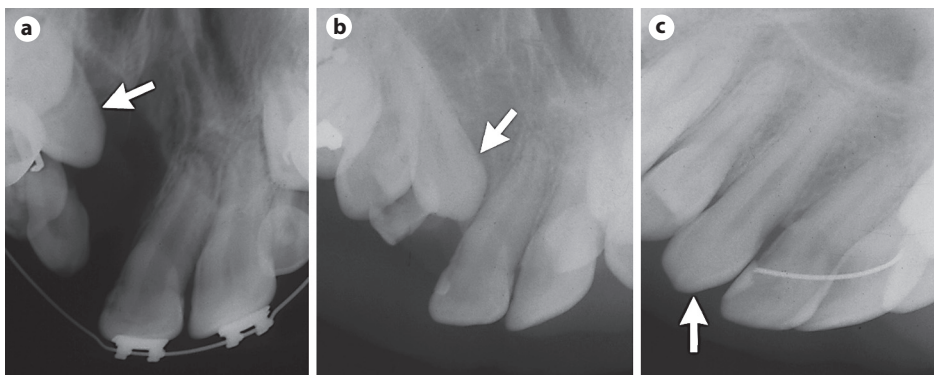


Fig. 2. Permanent canine eruption through a bone graft: (a) before alveolar bone grafting, (b) 2.6 years later, and (c) after orthodontic treatment.

However, when a substantial outward movement of the lateral segment(s) has been necessary, clinical experience suggests that bone grafting alone cannot be relied upon to maintain the expansion. In these circumstances, stabilisation in the form of a simple palatal arch would be advisable until the permanent dentition has erupted. Resting the patient from appliance therapy is highly desirable at this stage. Occasional observation is generally all that is necessary in the years between bone grafting and eventual eruption of the permanent dentition. The status of unerupted teeth, especially the cleft side canine, does however need careful monitoring.

Surgical Technique

Surgical technique varies in detail, especially with regard to flap design, but there is general agreement that muco-periosteal flaps including the attached gingiva should cover the marginal part of the graft [31, 43] (see fig. 1). This provides normal periodontal conditions around the teeth in the grafted region. It is important that the bony cleft is widely exposed to the level of the nasal cavity and that all scar tissue is removed. Every effort

should be made to avoid traumatizing the thin bone lamellae that cover the dental roots adjacent to the cleft. The nasal floor has to be carefully reconstructed when a fistula is present and most commonly cancellous bone from the iliac crest is packed high into the defect to give as much support to the alar base as possible. Meticulous closure of the nasal floor and suturing of the flaps without tension is important to avoid contamination from the nasal and/or oral cavity. In patients with bilateral clefts, both sides are normally operated at the same time [43].

Type of Donor Bone and Donor Site Morbidity

Many sources of bone, both autogenous and alloplastic, have been studied and compared, some highly profiled then abandoned, but fresh autologous cancellous bone is ideal because it supplies living immunocompatible bony cells to integrate fully with the maxilla and stimulate osteogenesis. The question of donor site has been debated for many years, with choice influenced by surgeon experience and preference, the volume of bone required, and the morbidity associated with the harvest [44]. The commonest donor sites are iliac

crest, cranium, mandibular symphysis, tibia and rib. The assumption that membranous bone is superior to endochondral was previously used to justify the use of cranial bone and mandibular bone, however this has not been confirmed [44].

Iliac crest: Bone from the iliac crest is easy to access and large quantities of cancellous bone containing osteogenic cells that can support osteogenesis in the early days of healing can be harvested. Because of its higher content of osteogenic cells, cancellous bone is superior to cortico-cancellous bone, and the process of compacting the bony chips into the defect is thought to increase its reliability further. Bone from the iliac crest can be harvested by an open approach or with a trephine. Some authors have reported concern with the trauma of traditional open harvesting and minimal invasive techniques have been suggested as alternatives [45, 46]. It is difficult to compare morbidity from different sites but the main criticism for using the iliac crest as a donor site is that postoperative pain would limit mobility and may require prolonged hospital stay. Two recent studies conclude that harvesting bone from the iliac crest appears to be well tolerated by patients, has few important complications, two teams can operate simultaneously which reduces operating time, gives aesthetically acceptable scars at the donor site and the hospital stay is a mean of 3 days [47, 48]. Indeed discharge after 24 h has been suggested provided the child can eat, drink and walk, the pain control is in place, and reliable carers are available [49, 50]. Iliac crest remains the most popular donor site, being favoured by 87% of European and 83% of North-American teams [29, 30].

Cranium: Both cortical and cancellous bone can be harvested from the calvarium in young patients and there are different harvesting techniques described. Several authors claim that the cranium is a donor site with low morbidity, minimal postoperative pain, a scar that is hidden in the hair, early discharge of the patient is possible and the outcomes are good [51–53]. However, some authors have reported poorer outcomes

when comparing cranial bone to bone from the iliac crest [54, 55]. Complications by harvesting cranial bone are relatively rare, the most common being haematomas or seromas and dural tear or exposure [54–56]. It is not possible for two teams to operate simultaneously when harvesting cranial bone, which lengthens the operation. Two percent of European and 8% of North-American teams use cranial bone for alveolar bone grafting [29, 30].

Mandibular symphysis: Good results of alveolar bone grafting with bone from the chin have been reported [38, 57–58], and being an intraoral site it is associated with short hospital stay, minimal pain and invisible scar. The main disadvantage of the symphysis as a donor site is the limited bone available, making it unsuitable for large bilateral clefts [57–59]. Some instances of damage to adjacent teeth, injury to the mental nerve and disturbance or sensitivity of adjacent teeth, and soft tissue have been reported [38, 57, 59]. It has been argued that bone grafts of cortico-cancellous bone (as from the chin) will give more cleft side canine impaction though this has been contradicted [38]. Four percent of European teams use chin as the donor site [29].

Tibia: Proponents of tibial bone report that there is enough bone, it is quick to harvest, has a short operation time, little blood loss, minimal scarring, and early mobility though patients should avoid contact sports for 3 weeks [32, 45, 60]. Others argue that the amount of bone available in tibia is limited and patients must be warned that it may be necessary to harvest from both legs [61, 62]. In young children the proximal tibia is small and the epiphyseal cartilage is growing, which means that access located inferiorly to avoid possible damage to a growth centre [60–62]. Three percent of European and 2% of North-American teams use tibia as donor site [29, 30].

Rib: Rib grafts are rarely used for alveolar bone grafting today, as the limitations of rib as donor site are: unsatisfactory amount of bone, risk of chest infections, unpleasant long-term discomfort

and possibly visible scars [58]. In Europe 0.5 and 3% of North-American teams use rib as the donor site [29–31].

Autogenous bony substitutes: Demineralised bone has been tried, but has limited use because unpredictability in resorption and the amount of bone that is formed [63].

Bone morphogenetic protein: In a systematic review of results of bone formation obtained by bone morphogenetic protein, van Hout et al. [64] identified three papers that compared BMP-2 with iliac crest bone grafting assessed by clinical and radiographic examinations [65–67]. Bone quality appeared comparable between the two methods in patients treated in the mixed dentition, and bone quantity appeared superior in the BMP-2 group in skeletally mature patients. However, the sample sizes are small and more research needs to be done to confirm the benefits of BMP-2. The advantages of using BMP-2 are shortening of operation time, absence of donor site morbidity, shorter hospital stay and reduction of overall cost [64].

Boneless-bone grafting: As noted above, Skoog introduced periosteoplasty in 1965 [17] and this procedure has been adopted by a few cleft teams in a modified form [68–70]. The proponents of gingivoperiosteoplasty state that if healthy periosteum is closed over the alveolar defect, favourable osteogenic conditions will allow bone to bridge it. Presurgical orthopaedics for 6 months preoperatively is usually necessary to align the maxillary segments before the surgery. This operation is usually done before the child is 2 years old and is reported to be a delicate operation, ‘not for the occasional operator’ [70]. The degree of ossification after gingivoalveoloplasty varies between 10 and 100% [69–71]. Matic and Power [71] in a large material with long-term follow-up reported poor outcome in 90% and that a failed procedure was detrimental to subsequent bone grafting. A high incidence of naso-alveolar fistula after gingivoalveoloplasty and poor facial growth were found [71–73]. Further reports on severe impairment of subsequent growth similar to that found

after primary bone grafting have been published and the technique has been abandoned in some centres [72–74]. One centre continues to use the technique despite growth disturbances as this is considered to be balanced by the avoidance of mixed dentition bone grafting [75].

Outcome Evaluation

Evaluation of bone grafts has traditionally been done on occlusal radiographs and several scales have been suggested [23, 26, 27, 76–78]. Some scales are almost identical, while others differ to the extent that comparisons between reports are impossible. The reproducibility of three of the published scales has been tested and found to be reliable [79]. The use of panoramic films to assess grafts is unreliable, while periapical films often fail to cover the field of interest. Radiographic assessment performed before full eruption of the teeth in the cleft site including the canine is especially unreliable.

Two-dimensional radiography in general appears to overestimate the extent of bony infill. Studies using three-dimensional computed tomography (3D CT) or three-dimensional cone-beam computed tomography (3D CBCT) have shown that especially in the bucco-palatal dimension there can be substantial bone loss after alveolar bone grafting [80–85]. Bone loss however appears significantly lower in patients with orthodontic space closure [82, 84, 85]. A large increase in bone volume when teeth erupt or are orthodontically moved into the bone-grafted region is observed [82, 84, 85] and the authors argue that the absolute figure of bone volume may not be so important. Clinically, the most important outcome is the presence of sufficient bone to allow orthodontic movement of teeth, survival of the teeth and a functional and aesthetic arch alignment. In 18 consecutive patients with orthodontic space closure assessed 20 years after bone grafting, 3D CT scans confirmed that there was full bony support

for all teeth in the cleft area for all patients, but there was less bone on the cleft side compared to the non-left side [86]. It is questionable whether 3D CT or 3D CBCT should be used regularly to assess pre- and post-surgery except where it is vital for further treatment planning.

Factors Influencing the Success of Alveolar Bone Grafting

Generally the results of mixed dentition bone grafting have been found to be good, with a high success rate [23–28, 35, 38, 76, 77, 87]. All authors have found that the result is better when bone grafting is performed before eruption of the permanent canine. A review of 992 grafted sites where the teeth adjacent to the cleft were in their final position, on consecutive patients treated by the Oslo cleft team, has been carried out by a panel of two internal and two external assessors. Good results (more than 3/4 of normal septum height) were found in 96% when the operation was done before canine eruption, and 85% when grafting was done after canine eruption [43]. A complete dental arch without having to resort to prosthetic restorations was found in 93%. Similar findings were reported by Lilja et al. [35] and Enemark et al. [38].

In a study of factors that influence the outcome of alveolar bone grafting, logistic regression analysis for 825 patients with 22 possible explanatory variables was performed [Semb, unpubl. data]. A panel of external and internal assessors used a modified Bergland scale to score the amount of bone in the interdental septum and in the naso-apical region. The most important factor associated with outcome was the individual surgeon who carried out the procedure, both for the interdental septum and the naso-apical area. Better outcome was observed in narrower clefts compared to large clefts, in clefts where the root of the canine was about half developed at the time of grafting, and when orthodontic space rather than prosthetic space closure was performed. It was worse when the lateral

incisor (from the smaller lateral segment) had been aligned. Surprisingly, better interdental bone fill were found in patients with bilateral rather than unilateral complete cleft lip and palate.

Canine Impaction

Canine impaction following bone grafting in patients with clefts is much higher than in children without clefts, the frequency ranging from 6 to 73% [24–26, 28, 38, 88–90] and is more frequent in unilateral than bilateral clefts [38, 88–90]. Semb and Schwartz [89] compared impaction rates in grafted patients and in patients with the same cleft types who were too old to receive grafts following the procedure's introduction. In the grafted group (n = 191) 25% of patients with UCLP had canine impaction compared with 14% of the non-grafted group (n = 80, p = 0.06). However, for BCLP, impaction occurred in 3% for the grafted (n = 88) and 6% for non-grafted (n = 50). As judged by 3D CBCT, neither the amount of root development nor the presence of the lateral incisor appeared to influence the direction of canine eruption [91].

Management of Space in the Dental Arch

The optimal treatment for missing lateral incisors (in about 45% of patients with alveolar clefts [92]) is a controversial issue and has been discussed at length in the orthodontic literature.

Orthodontic space closure: The major benefit in the author's opinion is that the end of the orthodontic treatment marks an end-point in major dental procedures for most patients. Re-contouring the canine to a more ideal lateral incisor shape and size following orthodontic space closure provides long-term results that are as good as, or superior to space opening for prosthetic replacement [93–95]. The periodontal conditions are significantly better with orthodontic space closure than with prosthetic replacement, the temporomandibular

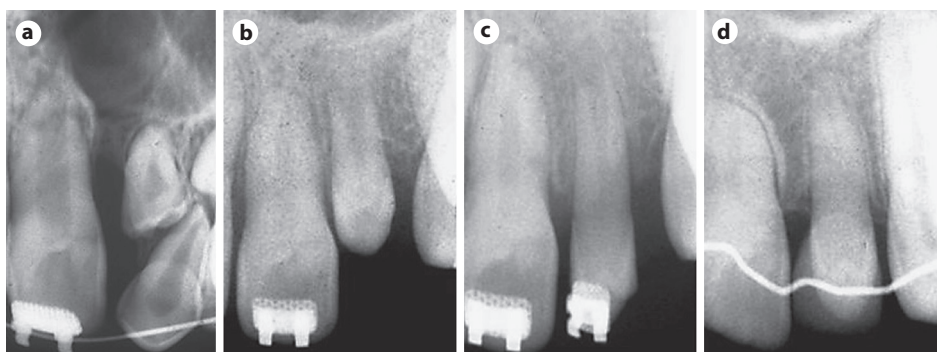


Fig. 3. Tooth transplantation into an alveolar bone graft: (a) before bone grafting, (b) 6 months after bone grafting, a small premolar from the non-cleft side has been transplanted into the bone graft, (c) 12 months after tooth transplantation, and (d) 12 years after tooth transplantation.

joint function is not impaired and patient satisfaction is high [4–6].

Tooth transplantation: Tooth transplantation is a good option in selected patients (fig. 3) who otherwise would have had to have a prosthesis and the procedure is a safe and reliable if done at an optimal time [96, 97]. The survival rate at a mean follow-up of 26.4 years (range 17–41 years) post-transplantation of 33 teeth in a Norwegian non-cleft sample was 90% [96]. Premolars in a crowded lower jaw are suitable candidates for transplantation to the upper arch. Periodontal and pulp healing is best achieved if transplantation is carried out when root development is half to three-quarters complete [96]. Experimental research suggests that simultaneous bone grafting and tooth transplantation should not be performed [98]. A 4- to 6-month period should be allowed for graft consolidation before tooth transplantation is done. Transplantation of ectopic teeth is also possible [40].

Space opening for prosthetic replacement: When the space in the cleft region is kept open for later prosthetic replacement this will lead to loss of the interdental alveolar bone height and thickness. The long-term maintenance/replacement of prostheses is a major disadvantage, and as noted above, periodontal health is less favourable.

Single tooth implants: Unfortunately, the maxillary lateral incisor region may be an unsuitable site for single tooth implant. A 10-year follow-up study of non-cleft patients with single tooth implants showed a progressive reduction of marginal bone level for the tooth adjacent to the implant [99]. The mean bone loss for the central incisor adjacent to the implant was 4.3 mm after 10 years. Other problems observed were gingival recessions, age changes in position of adjacent teeth, and the crown shape and colour of the implant crown.

However, many authors favour keeping the space open for implant placement in adulthood [100]. An additional bone grafting will generally be required. Implant placement immediately after bone grafting has a high risk of failure due to lack of stable anchorage. According to several reports the optimal time for implant placement should not exceed 6 months after augmentation bone grafting. If this is followed, the success rate is reported to be 70–99% at a follow-up time of 28–66 months [100–104]. One study with a control group showed that implant success was less in patients with cleft [103]. Marginal bone loss was not mentioned in two of these studies, and for the other two seemed quite substantial. The most important factor for successful implant placement seems to be limiting

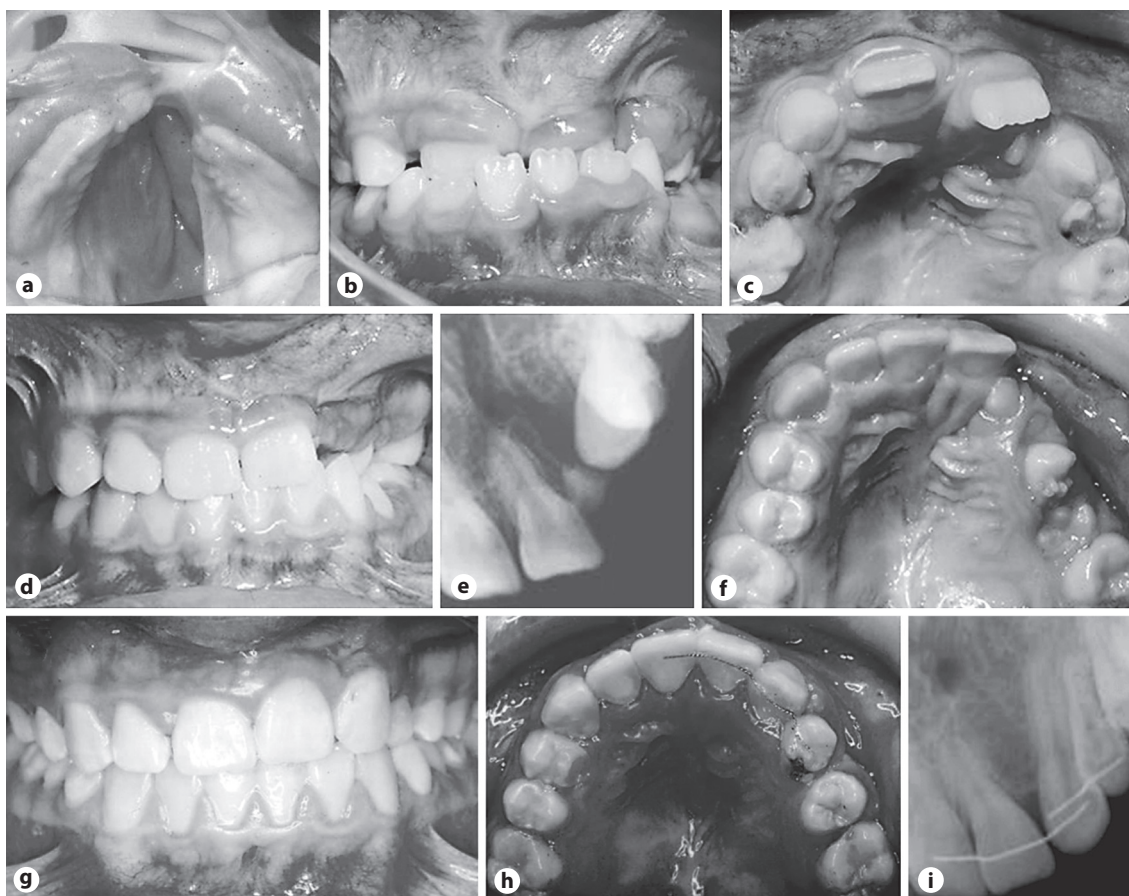


Fig. 4. Patient with unilateral cleft lip and palate: (a) at 3 months, before lip and palate closure, (b, c) anterior crossbite at 7 years of age, (d) after correction of the crossbite, (e) cleft site before grafting at 9.7 years (the lateral incisor is missing), (f) canine eruption following grafting, (g, h) 1.5 years after orthodontic treatment, and (i) 12.5 years following bone grafting.

the time between the bone grafting and the implant placement. Patients who have bone grafting in the mixed or early adult dentition and the space have been kept open for a later implant, should be regrafted using autogenous graft from the retro-molar area, mental symphysis or iliac crest. The implant is then placed 4–6 months later [100]. The length of the implant is significantly correlated to survival; the implant should be 13 mm or longer. In patients with alveolar clefts this is clearly more difficult to attain than in individuals without a cleft. Using implants to replace missing anterior

teeth must be considered carefully, taking account of possible gingival retraction, lack of interdental papilla and buccal alveolar bone loss leading to gingival discolouring, but especially the continuous bone loss along adjacent teeth.

The Future

For most cleft teams, alveolar bone grafting has become a core element in the management of patients with cleft involving the alveolus (fig. 4).

Indeed its introduction probably represents one of the most significant clinical innovations in cleft care in decades. Hopefully advances in tissue engineering will replace the need for transplantation

of autogenous bone in the future, or will provide an in-situ biological solution to the generation of a continuous bone fill across the alveolar cleft.

References

- Harvold E: Observations on the development of the upper jaw by harelip and cleft palate. *Odontol Tidskr* 1947;55:289–305.
- Bøhn A: Retention constructions, following Mr. Harvold's method of repositioning of the maxillary complex in cleft palate cases. *Eur Orthod Soc Rep* 1951:219–221.
- Ramstad T: Fixed prosthodontics in cleft palate; in McKinstry RE (ed): *Cleft Palate Dentistry*. Arlington, ABI Professional Publications, 1998, chap 9, pp 236–262.
- Nordquist GG, McNeill RW: Orthodontic vs. restorative treatment of the congenitally absent lateral incisor – long-term periodontal and occlusal evaluation. *J Periodontol* 1975;46:139–143.
- Andlin-Sobocki A, Eliasson LÅ, Paulin G: Periodontal evaluation of teeth in bone grafted regions in patients with unilateral cleft lip and palate. *Am J Orthod Dentofac Orthop* 1995;107:144–152.
- Robertsson S, Mohlin B: The congenitally missing upper lateral incisor. A retrospective study of orthodontic space closure versus restorative treatment. *Eur J Orthod* 2000;22:697–710.
- Von Eiselesberg FW: Zür Technik der Uranoplastik. *Arch Klin Chir* 1901;64:509–529.
- Lexer E: Die Verwendung der freien Knochenplastik nebst versuchen über Gelenkversteifung und Gelenktransplantation. *Acta Klin Chir* 1908;939–954.
- Koberg WR: Present view on bone grafting in cleft palate (a review of the literature). *J Oral Maxillofac Surg* 1973;1:185–193.
- Axhausen G: *Technik und Ergebnisse der Spaltplastiken*. München, Karl Hauser Verlag, 1952.
- Nordin KE: Treatment of primary total cleft palate deformity. Preoperative orthopaedic correction of the displaced components of the upper jaw in infants followed by bone grafting to the alveolar process clefts. *Trans Eur Orthod Soc Belfast* 1957;333–339.
- Schmid E: Die Annäherung der Kieferstümpfe bei Lippen-Kiefer-Gaumenspalten: Ihre schädlichen Folgen und Vermeidung. *Fortschr Kiefer Gesichtschir* 1955;1:168–173.
- Kriens O: Primary osteoplasty in patients with clefts of lip, alveolus and palate. *Acta Otorhinolaryngol Belg* 1968;22:687–696.
- Robertson NRE, Jolleys A: Effect of early bone grafting in complete clefts of the lip and palate. *Plast Reconstr Surg* 1968;42:414–421.
- Rehrmann AH, Koberg WR, Koch H: Long-term postoperative results after primary and secondary bone grafting in complete cleft lip and palate. *Cleft Palate J* 1970;7:206–221.
- Friede H, Johanson B: A follow-up study of cleft children treated with primary bone grafting. I. Orthodontic aspects. *Scand J Plast Reconstr Surg* 1974;8:88–103.
- Skoog T: The use of periosteal flaps in the repair of clefts in the primary palate. *Cleft Palate J* 1965;2:332–339.
- Brusati R, Mannucci N: The early gingivovulveoloplasty. *Scand J Reconstr Surg Hand Surg* 1992;26:65–70.
- Cutting C, Grayson B: The prolabial unwinding flap method for one-stage repair of bilateral cleft lip, nose and alveolus. *Plast Reconstr Surg* 1993;91:37–47.
- Boyne PJ, Sands NR: Secondary bone grafting of residual alveolar and palatal defects. *J Oral Maxillofac Surg* 1972;30:87–92.
- Boyne PJ, Sands NR: Combined orthodontic-surgical management of residual palato-alveolar cleft defect. *Am J Orthod* 1976;70:20–37.
- Albrektsson T: Healing of bone grafts; in vivo studies of tissue reactions at autografting in the rabbit tibia; thesis Gothenburg, Sweden 1979.
- Åbyholm F, Bergland O, Semb G: Secondary bone grafting of alveolar clefts. A surgical/orthodontic treatment enabling a non-prosthetic rehabilitation in cleft lip and palate patients. *Scand J Plast Reconstr Surg* 1981;15:127–140.
- Turvey TA, Vig K, Moriarty J, Hoke J: Delayed bone grafting in the cleft maxilla and palate: a retrospective multidisciplinary analysis. *Am J Orthod* 1984;86:244–256.
- Bergland O, Semb G, Åbyholm F: Elimination of the residual alveolar cleft by secondary bone grafting and subsequent orthodontic treatment. *Cleft Palate J* 1986;23:175–205.
- Enemark H, Sindet-Pedersen S, Bundgaard M: Long-term results of secondary bone grafting of alveolar clefts. *J Oral Maxillofac Surg* 1987;45:913–918.
- Lilja J, Friede H, Lauritzen C, Petterson L, Johanson B: Bone grafting at the stage of the mixed dentition in cleft lip and palate patients. *Scand J Plast Reconstr Surg* 1987;21:73–79.
- Paulin G, Åstrand P, Rosenquist JB, Bartholdson L: Intermediate bone grafting of alveolar clefts. *J Craniomaxillofac Surg* 1988;16:2–7.
- Shaw WC, Semb G, Nelson P, Brattström V, Mølsted K, Prah-Andersen B: *The Eurocleft Project 1996–2000. Standards for Care for Cleft Lip and Palate*. Amsterdam, IOS Press, 2000.
- Murthy AS, Lehman J: Evaluation of alveolar bone grafting: a survey of ACPA teams. *Cleft Palate Craniofac J* 2005;42:99–101.
- Turvey TA, Ruiz RL, Tiwana PS: Bone graft construction of the cleft maxilla and palate; in Losee JE, Kirschner RE (eds): *Comprehensive Cleft Care*. New York, McGraw-Hill, 2009, pp 837–865.

- 32 Walker TWM, Modayil PC, Cascarini L, Williams L, Duncan SM, Ward-Booth P: Retrospective review of donor site complications after harvest of cancellous bone from the anteriomedial tibia. *Br J Oral Maxillofac Surg* 2009;47:20–22.
- 33 Sillman MA: Dimensional changes of the dental arches: longitudinal study from birth to 25 years. *Am J Orthod* 1964;50:824.
- 34 Semb G: Effect of alveolar bone grafting on maxillary growth in unilateral cleft lip and palate patients. *Cleft Palate J* 1988;25:288–295.
- 35 Lilja J, Kalaaji A, Friede H, Elander A: Combined bone grafting and delayed closure of the hard palate in patients with unilateral cleft lip and palate: facilitation of lateral incisor eruption and evaluation of indicators for timing of the procedure. *Cleft Palate Craniofac J* 2000;37:98–105.
- 36 Ozawa T, Omura S, Fukuyama E, Matsui Y, Torikai K, Fujita K: Factors influencing secondary alveolar bone grafting in cleft lip and palate patients: prospective analysis using CT image analyzer. *Cleft Palate Craniofac J* 2007;44:286–291.
- 37 Precious DS: A new reliable method for alveolar bone grafting at about 6 years of age. *J Oral Maxillofac Surg* 2009;67:2045–2053.
- 38 Enemark H, Jensen J, Bosch C: Mandibular bone graft material for reconstruction of alveolar cleft defects: long-term results. *Cleft Palate Craniofac J* 2001;38:155–163.
- 39 Sindet-Pedersen S, Enemark H: Management of impacted teeth in congenital clefts; in Alling CC, Helfrick JF, Alling RD (eds): *Impacted Teeth*. Philadelphia, Saunders, 1993, pp 344–352.
- 40 Semb G, Shaw WC: *Orthodontics*; in Watson A, Sell D, Grunwell P (eds): *Management of Cleft Lip and Palate*. London, Whurr Publishers, 2001, chapt 19, pp 299–325.
- 41 Semb G, Rønning E, Åbyholm FE: Twenty years follow-up of 50 patients with unilateral cleft lip and palate. *Semin Orthod* 2011;17:207–224.
- 42 Vargervik K: Orthodontic treatment of cleft patients: Characteristics of growth and development/treatment principles; in Bardach J, Morris HL (eds): *Multidisciplinary Management of Cleft Lip and Palate*. Philadelphia, Saunders, 1990, chapt 78, pp 642–649.
- 43 Åbyholm FE: Secondary bone grafting of alveolar clefts; in Berkowitz S (ed): *Cleft Lip and Palate. Diagnosis and Management*. Berlin, Springer, 2006, pp 601–605.
- 44 Rawashdeh MA, Telfah H: Secondary alveolar bone grafting: the dilemma or donor site selection and morbidity. *Oral Maxillofac Surg* 2008;46:665–670.
- 45 Ilankovan V, Stronczek M, Telfer M, Peterson W, Strassen LF, Ward-Booth P: A prospective study of trephine bone grafts of the tibial shaft and iliac crest. *Br J Oral Maxillofac Surg* 1998;36:434–439.
- 46 Sharma S, Schneider LF, Barr J, Aarabi S, Chibbaro P, Grayson B, Cutting CB: Comparison of minimally invasive versus conventional open harvesting technique for iliac bone graft in secondary alveolar cleft patients. *Plast Reconstr Surg* 2011;128:485–491.
- 47 Swan MC, Goodacre TE: Morbidity at the iliac crest donor site following bone grafting of the cleft alveolus. *Br J Oral Maxillofac Surg* 2006;44:129–133.
- 48 Baqain ZH, Anabtawi M, Karaky AA, Malkawi Z: Morbidity from anterior iliac crest bone harvesting for secondary alveolar bone grafting: an outcome assessment study. *J Oral Maxillofac Surg* 2009;67:570–575.
- 49 Rudman RA: Prospective evaluation of morbidity associated with iliac crest harvest for alveolar cleft grafting. *J Oral Maxillofac Surg* 1997;55:219–224.
- 50 Perry CW, Lowenstein A, Rothkopf DM: Ambulatory alveolar bone grafting. *Plast Reconstr Surg* 2005;116:736–740.
- 51 Wolfe SA, Berkowitz S: The use of cranial bone in the closure of alveolar and anterior palatal clefts. *Plast Reconstr Surg* 1983;72:659–671.
- 52 Jackson IT, Helden G, Marx R: Skull bone grafts in maxillofacial and craniofacial surgery. *J Oral Maxillofac Surg* 1986;44:949–955.
- 53 Denny AD, Talisman R, Bonawitz SC: Secondary alveolar bone grafting using milled cranial bone graft: a retrospective study of a consecutive series of 100 patients. *Cleft Palate Craniofac J* 1999;36:144–153.
- 54 Kortebein MJ, Nelson CL, Sadove AM: Retrospective analysis of 135 secondary alveolar cleft grafts using iliac or calvarial bone. *J Oral Maxillofac Surg* 1991;49:493–498.
- 55 LaRossa D, Buchman S, Rothkopf DM, Mayo R, Randall PA: A comparison of iliac and cranial bone in secondary grafting of alveolar clefts. *Plast Reconstr Surg* 1995;96:789–799.
- 56 Kline RM Jr, Wolfe SA: Complications associated with the harvesting of cranial bone grafts. *Plast Reconstr Surg* 1995;95:5–20.
- 57 Sindet-Pedersen S, Enemark H: Reconstruction of alveolar clefts with mandibular or iliac crest bone grafts: a comparative study. *J Oral Maxillofac Surg* 1990;48:554–560.
- 58 Freihofer HPM, Borstlap WA, Kuijpers-Jagtman AM, Voorsmit RACA, van Damme PA, Heidebuchel KLWM, Borstlap-Engels VMF: Timing and transplant materials for closure of alveolar clefts. A clinical comparison of 296 cases. *J Craniomaxillofac Surg* 1993;21:143–148.
- 59 Booij A, Raghoobar GM, Jansma J, Kalk WWI, Vissink A: Morbidity of chin bone transplants used for reconstructing alveolar defects in cleft patients. *Cleft Palate Craniofac J* 2005;42:533–538.
- 60 Kalaaji A, Lilja J, Elander A, Friede H: Tibia as donor site for alveolar bone grafting in patients with cleft lip and palate: long-term experience. *Scand J Plast Reconstr Surg Hand Surg* 2001;35:35–42.
- 61 Hughes CW, Revington PJ: The proximal tibia donor site in cleft alveolar bone grafting: experience of 75 consecutive cases. *J Craniomaxillofac Surg* 2002;30:12–17.
- 62 Chen YC, Chen CH, Chen PL, Huang IY, Shen YS, Chen CM: Donor site morbidity after harvesting of proximal tibia bone. *Head Neck* 2006;28:496–500.
- 63 Horswell BB, Henderson JM: Secondary osteoplasty of the alveolar cleft defect. *J Oral Maxillofac Surg* 2003;61:1082–1090.
- 64 Van Hout WMMT, van der Molen ABM, Breugem CC, Koole R, van Cann EM: Reconstruction of the alveolar cleft: can growth factor-aided tissue engineering replace autologous bone grafting? A literature review and systematic review of results obtained with bone morphogenetic protein-2. *Clin Oral Invest* 2011;15:297–303.
- 65 Herford AS, Boyne PJ, Rawson R, Williams RP: Bone morphogenetic protein-induced repair of the premaxillary cleft. *J Oral Maxillofac Surg* 2007;65:2136–2141.

- 66 Dickinson BP, Ashley RK, Wasson KL, O'Hara C, Gabbay J, Heller JB, Bradley JP: Reduced morbidity and improved healing with bone morphogenetic protein-2 in older patients with alveolar defects. *Plast Reconstr Surg* 2008;121:209–217.
- 67 Alonso N, Tanikawa DY, Freitas RD, Canan L, Ozawa TO, Rocha DL: Evaluation of maxillary reconstruction using a resorbable collagen sponge with recombinant human bone morphogenetic protein-2 in cleft lip and palate patients. *Tissue Eng Part C Methods* 2010;16:1183–1189.
- 68 Santiago PE, Grayson BH, Cutting CB, Gianoutsos MP, Brecht LE, Kwon SM: Reduced need for alveolar bone grafting by presurgical orthopedics and primary gingivovuloplasty. *Cleft Palate Craniofac J* 1998;35:77–80.
- 69 Meazzini MC, Tortora C, Morabito A, Garattini G, Brusati R: Alveolar bone formation in patients with unilateral and bilateral cleft lip and palate after early secondary gingivovuloplasty: long-term results. *Plast Reconstr Surg* 2007;119:1527–1537.
- 70 Cutting CB, Grayson BH: The effects of gingivovuloplasty following alveolar molding with pin-retained Latham appliance versus secondary bone grafting on midfacial growth in patients with unilateral clefts. *Plast Reconstr Surg* 2008;121:871–873.
- 71 Matic DB, Power SM: Evaluating the success of gingivovuloplasty versus secondary bone grafting in patients with unilateral clefts. *Plast Reconstr Surg* 2008;121:1343–1353.
- 72 Matic DB, Power SM: The effects of gingivovuloplasty following alveolar molding with a pin-retained Latham appliance versus secondary bone grafting on midfacial growth in patients with unilateral clefts. *Plast Reconstr Surg* 2007;122:863–870.
- 73 Power SM, Matic DB: Gingivovuloplasty following alveolar molding with Latham appliance versus secondary bone grafting: the effect on bone production and midfacial growth in patients with bilateral clefts. *Plast Reconstr Surg* 2009;124:573–582.
- 74 Henkel K-O, Gundlach KKH: Analysis of primary gingivovuloplasty in alveolar clefts repair. I. Facial growth. *J Craniomaxillofac Surg* 1997;25:266–269.
- 75 Meazzini M, Rossetti G, Garattini G, Semb G, Brusati R: Early secondary gingivovuloplasty in the treatment of unilateral cleft lip and palate patients: 20 years' experience. *J Craniomaxillofac Surg* 2010;38:185–194.
- 76 Long RE, Spangler BE, Yow M: Cleft width and secondary alveolar bone graft success. *Cleft Palate Craniofac J* 1995;32:420–427.
- 77 Kindelan JD, Nashed RR, Bromige MR: Radiographic assessment of secondary autogenous alveolar bone grafting in cleft lip and palate patients. *Cleft Palate Craniofac J* 1997;34:195–198.
- 78 Witherow H, Cox S, Jones E, Carr R, Waterhouse N: A new scale to assess radiographic success of secondary alveolar bone grafts. *Cleft Palate Craniofac J* 2002;39:255–260.
- 79 Nightingale C, Witherow H, Reid FD, Edler R: Comparative reproducibility of three methods of radiographic assessment of alveolar bone grafting. *Eur J Orthod* 2002;25:35–41.
- 80 Rosenstein SW, Long RE, Dado DV, Vinson B, Alder ME: Comparison of 2D calculations from periapical and occlusal radiographs versus 3D calculations from CAT scans in determining bone support for cleft-adjacent teeth following early alveolar bone grafts. *Cleft Palate Craniofac J* 1997;34:199–205.
- 81 Van der Meij AJW, Baart JA, Prah Andersen B, Valk J, Kostense PJ, Tuinzing DB: Bone volume after secondary bone grafting in unilateral and bilateral clefts determined by computed tomography scans. *Oral Surg Oral Med Oral Pathol* 2001;92:136–141.
- 82 Schultze-Mosgau S, Nkenke E, Schlegel AK, Hirschfelder U, Wiltfang J: Analysis of bone resorption after secondary alveolar bone grafts before and after canine eruption in connection with orthodontic gap closure or prosthodontic treatment. *J Oral Maxillofac Surg* 2003;61:1245–1248.
- 83 Iino M, Ishi H, Matsushima R, Masayuki F, Hamada Y, Kondoh T, Seto K: Comparison of intraoral radiography and computed tomography in evaluation of formation of bone after grafting for repair of residual alveolar defects in patients with cleft lip and palate. *Scand J Plast Surg Hand Surg* 2005;39:15–21.
- 84 Feichtinger M, Mossböck R, Rärcher H: Assessment of bone resorption after secondary alveolar bone grafting using three-dimensional computed tomography: a three year study. *Cleft Palate Craniofacial J* 2007;44:142–148.
- 85 Oberoi S, Chigurupati R, Pawandeep G, Hoffman WY, Vargervik K: Volumetric assessment of secondary alveolar bone grafting using cone beam computed tomography. *Cleft Palate Craniofac J* 2009;46:503–511.
- 86 Kolbenstvedt A, Aaløkken TM, Arctander K, Johannesen S: CT appearances of unilateral cleft palate 20 years after bone graft surgery. *Acta Radiol* 2002;43:567–570.
- 87 Trindade IK, Mazzottini R, da Silva Filho OG, Trindade IEK, Deboni MCZ: Long-term radiographic assessment of secondary alveolar bone grafting outcomes in patients with alveolar clefts. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2005;100:271–277.
- 88 El Deeb M, Messer LB, Lehnert MW, Hebda TW, Waite DE: Canine eruption into grafted bone in maxillary alveolar cleft defects. *Cleft Palate J* 1982;19:9–16.
- 89 Semb G, Schwartz O: The impacted tooth in patients with alveolar clefts; in Andreasen JO, Petersen JK, Laskin DM (eds) *Textbook and Color Atlas of Tooth Impaction*. Copenhagen, Munksgaard, 1997, chapt 12, pp 331–348.
- 90 Matsui K, Echigo S, Kimizuka S, Takahashi M, Chiba M: Clinical study on eruption of permanent canines after secondary alveolar bone grafting. *Cleft Palate Craniofac Surg* 2005;42:309–313.
- 91 Oberoi S, Gill P, Chigurupati R, Hoffman WY, Hatcher DC, Vargervik K: Three-dimensional assessment of the eruption path of the canine in individuals with bone-grafted alveolar clefts using cone beam computed tomography. *Cleft Palate Craniofac J* 2010;47:507–512.

- 92 Böhn A: Dental anomalies in harelip and cleft palate. *Acta Odontol Scand* 1963;21:1–109.
- 93 Rosa M, Zachrisson BU: Integrating esthetic dentistry and space closure in patients with missing maxillary lateral incisors. *J Clin Orthod* 2001;35:221–234.
- 94 Tuverson DL: Close space to treat missing lateral incisors. *Am J Orthod* 2004;125:17A.
- 95 Zachrisson BU: Improving the esthetic outcome of canine substitution for missing maxillary lateral incisors. *World J Orthod* 2007;8:72–79.
- 96 Czochrowska EM, Stenvik A, Bjercke B, Zachrisson BU: Outcome of tooth transplantation: survival and success rates 17–41 years' post-treatment. *Am J Orthod Dentofac Orthop* 2002;121:110–119.
- 97 Czochrowska EM, Semb G, Stenvik A: Non-prosthetic management of alveolar cleft with two incisors missing on the cleft side. A report of 5 cases. *Am J Orthod Dentofac Orthop* 2002;122:587–592.
- 98 Stenvik A, Semb G, Bergland O, Åbyholm F, Beyer-Olsen EMS, Gerner N, Haanæs HR: Experimental transplantation of teeth to simulated maxillary alveolar clefts. *Scand J Plast Reconstr Surg* 1989;23:105–108.
- 99 Thilander B, Odman J, Lekholm U: Orthodontic aspects of the use of oral implants in adolescents: a 10-year follow-up study. *Eur J Orthod* 2001;23:715–731.
- 100 Pena WA, Vargervik K, Sharma A, Oberoi S: The role of implants in the management of alveolar clefts. *Pediatr Dent* 2009;31:329–333.
- 101 Kearns G, Perrott DH, Sharma A, Kaban LB, Vargervik K: Placement of endosseous implants in grafted alveolar clefts. *Cleft Palate Craniofac J* 1997;34:520–525.
- 102 Härtel J, Pögl C, Henkel K-O, Gundlach KKH: Dental implants in alveolar cleft patients: a retrospective study. *J Craniomaxillofac Surg* 1999;27:354–357.
- 103 Kramer F-J, Baethge C, Swennen G, Bremer B, Schwestka-Polly R, Dempf R: Dental implants in patients with orofacial clefts: a long-term follow-up study. *Int J Oral Maxillofac Surg* 2005;34:715–721.
- 104 Matsui Y, Ohno K, Nishimura A, Shiota T, Kim S, Miyashita H: Long-term study of dental implants placed into alveolar cleft sites. *Cleft Palate Craniofac J* 2007;44:444–447.

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Speech and Language in the Patient with Cleft Palate

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Abstract

This chapter describes the normal development of speech and speech problems that may arise for the child born with cleft lip and/or palate. It describes current trends and the importance of multidisciplinary working in this complex field. The contribution of the speech and language therapist to the management of this population is considered.

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The multidisciplinary treatment of children born in the UK with cleft lip and/or palate (CLP) has undergone transformation since the publication of the CSAG report in 1998 [1]. Twelve cleft centres have been established and all children born with facial clefts are enrolled from diagnosis in a 20-year programme of multidisciplinary treatment. Teams have been established in all the centres and clinicians work in a coordinated and unified way. The development of a gene bank and clinical studies group associated with the Healing Foundation will encourage a much more national and systematic approach to research. Increasingly, centres are auditing the outcomes of their treatment and sharing their results nationally.

Speech is considered to be one of the primary measures of outcome in cleft palate management [1]. All children born with a cleft palate must

therefore receive specialist speech assessments and therapy recommendations made where appropriate. The surgery that children undergo to repair their clefts is carried out in the centres but many children live remotely from these centres. Therefore, outreach clinics are often run throughout the network so that ongoing multidisciplinary care can be carried out nearer to the child's home. Speech and language therapy, where needed, should be carried out locally, so that liaison with other services can be effectively managed. Unfortunately, waiting lists in community clinics where children receive their therapy can be long and care is not universally available on a regular basis from a qualified speech and language therapist.

Development of Speech and Language

The development of mature speech and language that is easily understood by family and others is a major developmental feature of the first 5 years of a child's life. It relies on the successful integration of many different systems – neurological, auditory, and a nurturing environment. Children mature at different speeds. There is a hierarchy of order in speech and language development but

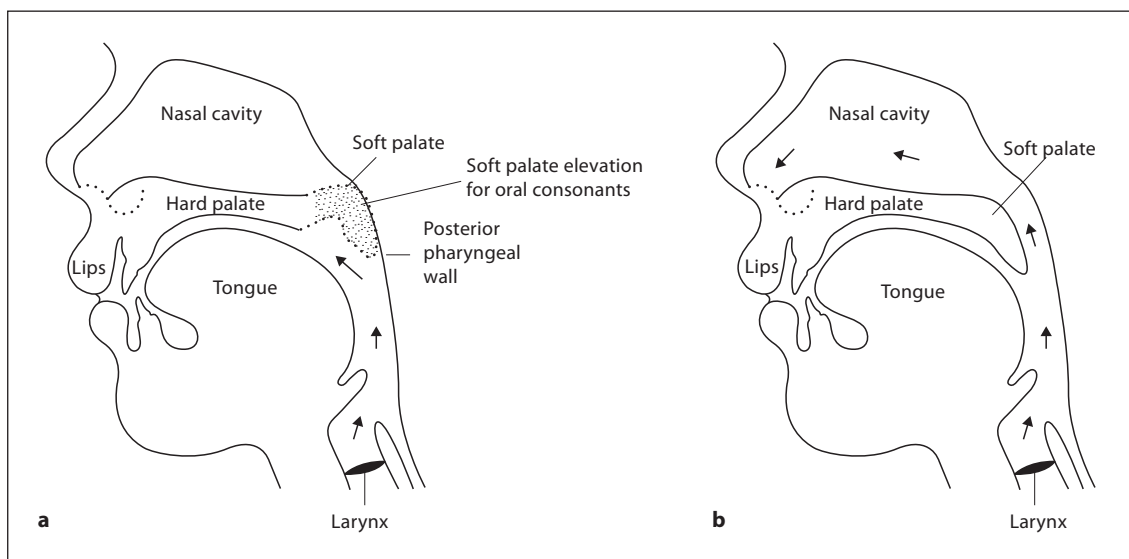


Fig. 1. Palatal function during speech. **a** The palate is raised to the posterior pharyngeal wall during oral consonant and vowel production. **b** The palate is lowered at rest and during nasal consonant production shows.

much variation will be seen in the normally developing child as well as in any child with a developmental difficulty. Communication starts with the first cry of the newborn baby. Meaning is assigned to the different cries that a baby makes by the actions of its mother (feeding when thought to be hungry, changing a nappy). Gurgling, cooing and then babbling (repetition of syllable strings) follow during the first year of life. Early single words are usually recognised by the parents at around 12 months of age and single word vocabulary increases rapidly until the child gradually links the words into phrases (18–24 months) and sentences (2–3 years). There is a wide variation between individuals and slower language development is not necessarily a predictor of long-term problems.

Normal Speech

Speech is produced on pulmonary egressive air passing up through the larynx into the nasal and

oral cavities. Within the mouth, the airstream is modified by the movement of the tongue and lips into the consonants and vowels that make up the speaker's language. The velopharyngeal orifice is the opening between nose and mouth and is a sphincter formed by muscles of the pharynx and soft palate. The velum (soft palate) rises and reaches the posterior pharyngeal wall. Simultaneous inward movements of the lateral pharyngeal and posterior pharyngeal walls combine to close the velopharyngeal sphincter. This closure directs the ascending airstream into the mouth where the oral consonants [p], [b], [t], [d], [k], [g], [s], [z], [sh], [ch] and vowels are shaped (fig. 1a). For nasal consonants [m], [n], [ŋ] and their adjacent vowels, the velum remains lowered, allowing the air to pass into the nasal cavity (fig. 1b). This velopharyngeal mechanism must be capable of very rapid movements to produce normally balanced tone and voice.

Potential Barriers to Speech Development for a Child Born with Cleft Palate

The aetiology of speech disorders for the child with cleft palate is often multifactorial and complex. The nature of surgery, the timing of the palate repair, surgical skill and availability of speech and language therapy all interact and can contribute to ongoing speech problems [2]. Periods of fluctuating hearing loss, common in this population, present additional challenges to speech and language development. Acceptable speech is one goal of surgical treatment for cleft palate and is a major outcome along with facial growth and normal feeding [1]. In the child with cleft palate, various studies have described delayed development of babbling and a tendency to posterior placements of their tongue [3–5]. These features may continue to impact on speech development as there is a recognised tendency to hold the tongue further back in the mouth during speech in an individual born with cleft palate [6]. Babies start deliberate repetition of consonant sounds (babbling) at around 6 months. Could earlier cleft palate repair improve the speech outcome by providing the infant with a more normal anatomy before too much babbling takes place? A NIH-funded trial of the timing of surgery for cleft palate is currently taking place and this will look at a range of outcomes, including speech [7].

Anatomy

The baby born with a cleft palate is at an immediate disadvantage for their speech development because of the anatomical difference created by the connection between oral and nasal cavities. Most babies born in the UK receive their cleft palate repair before the age of 1 year. Delaying the palate repair beyond this age is usually as a result of an infant's additional health problems, which will also impact on speech and language

development. In addition to overt forms of cleft palate there exists a condition known as submucous cleft palate. The mucous membrane covering the soft palate is intact but the underlying muscles may be misplaced. This condition may not be recognised unless it is symptomatic, either because there is significant nasal regurgitation of milk, or later when speech develops. It may also be a chance discovery when presenting for other medical or dental examinations. The physical signs of submucous cleft palate are variable. There may be a bifid uvula, a translucent zone along the midline of the soft palate caused by muscle diastasis or a notch palpable at the border of the hard and soft palates. No surgical treatment is indicated if the signs are present but there is an absence of symptoms, such as hypernasal speech or nasal regurgitation of milk [8].

Hearing

All children born with cleft palate are at considerable risk of fluctuating hearing loss. Studies have shown that eustachian tube dysfunction and middle ear effusions are widespread amongst this population [9]. It was hoped that early repair of the palate would restore function, however this appears not to be a universal finding. During the development of speech hearing loss can have a profound effect on speech sound acquisition and the child's ability to benefit from speech therapy. Children with repaired cleft palate therefore need to receive ongoing monitoring of their hearing and active management of any problem. There is a trend away from universal insertion of grommets (ventilation tubes) at the time of palate repair although they may be inserted when middle ear effusions persist.

Syndromes

The presence of a syndrome may also have a great impact on the development of speech and

language in an infant. There is a significant association between isolated cleft palate and syndromes. Shprintzen et al. [10] reported 53% of a consecutive series of 1,000 patients with cleft lip and palate from the Montefiore Center in New York having a syndrome in addition to their cleft diagnosis. One of the syndromes most frequently seen by the cleft palate team is velocardiofacial syndrome, also known as 22q11 deletion syndrome. This is a complex syndrome with great variation in presentation and severity. Major cardiac anomalies are often treated during the early months of life. The child may have a cleft palate or non-cleft velopharyngeal dysfunction (VPD). Language and speech development are likely to be significantly delayed. Learning difficulties and many other problems may be associated with this syndrome [11]. There are many other syndromes associated with cleft lip and/or palate but those which may have a particular impact on speech and language development include trisomy 13, trisomy 18, CHARGE association, Goldenhar syndrome, Treacher Collins syndrome and Kabuki syndrome. It is important to recognise that if there is a syndrome present, the most appropriate interventions should be offered and a realistic view of possible outcomes maintained.

Incidence of Speech Problems for a Child Born with Cleft Palate

Many differences in prevalence of speech difficulties will be found in the literature. One of the main reasons is that there has not been a widely recognised and accepted tool of measurement. Spriestersbach et al. [5] and Stengelhofen [12] have quoted speech difficulties for 50% of children born with cleft palate. In contrast, Hall and Golding-Kushner [13] have quoted a better achievement – 80% of children having speech which does not require therapeutic intervention.

Assessment of Speech in the Child with Cleft Palate

Within the UK, great progress has been made creating an acceptable and widely used tool of speech assessment and more recently, a tool for the audit of speech outcomes. The Great Ormond Street Speech Assessment Framework for Cleft Palate and Velopharyngeal Anomalies (GOS.SP.ASS) [14, 15] is the nationally agreed tool for assessment and admirably provides speech and language therapists with a systematic framework for their use in assessment and diagnosis of speech difficulties associated with cleft palate. A further development – The Speech Audit Tool – ‘Cleft Audit Protocol for Speech – Augmented’ (CAPS-A) [16] has been adopted and this has allowed cross-centre and even national collaborations in audit. Consensus listening exercises are carried out on recordings of children’s speech, allowing the collection of outcome data. These structured listening exercises are intended to remove the possibility of bias in the single listener – particularly if they are familiar with the child. One of the problems in describing speech outcome is the complex nature of communication. To have a single outcome measure is an unachievable aspiration. The terms ‘intelligibility’ or ‘acceptability’ of speech mean subtly different things to different people. In addition, they are affected by too many uncontrollable variables sometimes also involving the listener. Both GOS.SP.ASS and CAPS-A use a structured approach to judgements by dividing the assessment into different tasks. These include listening to spontaneous speech, rote speech (counting, days of the week) and specific speech-sound repetition. Using GOS.SP.ASS, the therapist can analyse speech difficulties, allowing them to determine the cause of the speech problem and plan therapy or wider management.

Speech and language therapists throughout the UK are currently testing some process and outcome standards for speech in the child with

repaired cleft palate. These standards have been tested on children with non-syndromic CLP. Outcome standard 1 states that at the age of 5 to 5 years 11 months, 70% of children born with cleft palate should have speech within the normal range. UK cleft specialist speech and language therapists believe this should be achievable. Standard 2 is that fewer than 50% of children will have a serious articulation (pronunciation) problem. This is being achieved [17].

Characteristics of Cleft Palate Speech

How should we describe cleft palate speech? The skill of the speech and language therapist is to diagnose the focus and severity of the difficulty and provide the appropriate treatment for a child with a repaired cleft palate. The speech problems relating to cleft palate will generally fall into the following categories: abnormal nasal resonance, nasal emission or turbulence and pronunciation difficulties known as cleft speech characteristics (CSCs) [18].

Abnormal Nasal Resonance

If there is a resonance problem it is most common to hear hypernasality – the sound of excess air leaking into and resonating around the nasal cavity. It may be categorised as mild, moderate or severe, or more commonly now given a numerical value on the GOS.SPASS framework. Mild hypernasality is perceived mainly on vowels. Severe hypernasality may result in the substitution of a target oral sound such as [b] or [d] with their nasal counterpart so that [b] becomes [m] or [d] becomes [n]. Hyponasality may be heard. This is a reduction in nasal resonance making the speaker sound congested. This is not such an unusual sound occurring as it does reasonably frequently in speakers without cleft palate and anyone suffering from an upper respiratory infection. Occasionally one hears a combination of hyper- and hyponasality.

Abnormal Nasal Airflow

Nasal air emission refers to the sound of air escaping down the nose accompanying a speech sound and is a result of abnormal airflow, either passing around the back of the soft palate into the nose or through an oro-nasal fistula in the hard or soft palate, which may remain after palate surgery. Nasal turbulence is also caused by abnormal airflow and may be heard as a snorting noise, which accompanies a speech sound. Both nasal air emission and nasal turbulence can be loud and distracting or quiet and mainly detected by a trained speech and language therapist. Abnormal voice is also sometimes described. The voice may sound weak, or present with low volume or the voice may sound dysphonic (hoarse, husky or strained). Problems of resonance and airflow point to a probable diagnosis of VPD, which will be discussed in more depth later in the chapter.

Cleft Speech Characteristics

As a result of assessing a child's speech, a differential diagnosis should be made between speech errors associated with the cleft palate, those caused by developmental immaturity which should resolve in time and any other speech sound disorders. Sound difficulties associated with cleft palate can be summarised as anterior, posterior, non-oral, or passive cleft speech characteristics [15, 16].

Anterior Cleft Speech Characteristics

These are sound difficulties made in the anterior part of the mouth. Sounds such as [t], [d], [s], and [z] may have a slushy or lisping quality. There may be a tendency to protrude the tongue beyond the teeth (interdentalisation). This difficulty can also be caused by an element of immaturity and may therefore improve spontaneously over time. However, children with CLP where the cleft affects the alveolus will often have dental irregularities

which persist for some time. They will often have an ongoing tendency to a palatal or lateral lisping quality to their speech [19]. Therapy can help change these patterns if a child is motivated but these subtle differences may remain and can be difficult to change.

Posterior Cleft Speech Characteristics

These are more serious sound difficulties produced further back in the mouth. Children born with a cleft palate often hold their tongue in a more retracted position in the mouth. The resulting sound difficulties may mean that they pronounce [t] as [k] so for 'tea' they may say 'key'. As a rule the child with this sort of problem will be fairly systematic in their difficulty so that other sounds such as [d], [s] and [z] might also be affected making speech quite difficult to understand. A residual oro-nasal fistula might also encourage this retracted pattern. Therapy is needed to teach the child to change these speech patterns. The speech and language therapist might also request that the fistula be closed if it is felt that this is preventing the child from changing their speech pattern. A period of 'diagnostic' or 'trial' therapy to see what changes can be made is often carried out.

Non-Oral Cleft Speech Characteristics

These are sound errors made outside the oral cavity in the pharynx and glottal area. They often develop in a child whose velopharyngeal mechanism is not working adequately or are habits which developed during the period when the palate was inadequate, even though a repair has now been successfully carried out. Vulnerable sounds are often [p], [b], [t], [d], [s], [z], [sh], [k], [g]. If a glottal stop is substituted for many of these sounds, the speech will be very difficult to understand. The management of these sound difficulties will include therapy to alter speech patterns if the palate is closing adequately or a combination of surgery and therapy if there is ongoing VPD. The child may find therapy difficult and

changing some of these speech features can take some time.

Passive Cleft Speech Characteristics

These speech sound errors are caused by an inadequate velopharyngeal mechanism and can be very severe and impact a great deal on intelligibility. The patient may try to produce the correct sound by placing his tongue in the correct position but air floods into the nasal cavity and the sound might come out as its nasal equivalent. Thus, [d] becomes [n] so that attempts at the word 'daddy' sound like 'nanny' or the sounds may be nasalised, giving the child's speech a rather hypernasal 'twang'. The management of these difficulties is to investigate the need for further surgery, known as 'secondary speech surgery'. A child with this difficulty is said to have 'velopharyngeal dysfunction'. Speech therapy treatment cannot overcome VPD. Here the role of the therapist is to identify the problem and to ensure the child has further assessment of VPD, generally in conjunction with the cleft surgeon.

Velopharyngeal Dysfunction

VPD is an umbrella term which can be defined as a problem in which the sphincter created by the combination of lift of the soft palate to the posterior pharyngeal wall and the inward movements of the posterior and lateral pharyngeal walls fails to close completely. This causes air and sometimes liquid or foodstuff to leak into the nose inappropriately. Velopharyngeal insufficiency implies a shortage of tissue as a cause and velopharyngeal inadequacy implies a poorly moving palate. In practice, these terms are often used interchangeably [20]. In the CSAG report the incidence of current or previously treated VPD was 27% in 5-year-olds. There is wide variation in the literature – authors stating a range between 5 and 40% of children with repaired cleft palate showing signs of VPD [2, 21].

Management of Velopharyngeal Dysfunction

Children with repaired cleft palate undergo speech assessment regularly during their pre-school years. Early signs of velopharyngeal problems such as hypernasality, a predominance of nasal consonants [m] and [n], or glottal stop production in place of early developing sounds such as [p], [b], [t], [d], [k], [g], will alert the speech and language therapist to the possibility of VPD. The child may also have ongoing nasal regurgitation of fluids or food. The child with these features should be monitored regularly and the therapist will try to see if they are capable of targeting the correct sounds. They may undertake some 'diagnostic' therapy sessions, which will help to make the differential diagnosis between speech sound difficulties that may improve with therapy and maturation and VPD where the child needs further surgery to allow normal speech to develop. When the speech and language therapist is confident that the child has suspected VPD the child will attend a velopharyngeal investigation clinic. The investigations include a detailed speech assessment to show the symptoms of VPD and a videofluoroscopy examination. This is a radiograph with speech, where movements of the palate during a speech sample are recorded. A lateral view is routinely captured. Sometimes multiple views are also taken. In some cases, nasendoscopy is carried out to give additional information by viewing the velopharyngeal orifice from above. Following these investigations the surgeon, speech and language therapist and family can plan the next step. This is likely to be further surgery, possibly in combination with more speech therapy treatment.

Instrumentation

Perceptual assessment of speech is the most valuable tool used in the evaluation of speech outcomes related to cleft palate [22]. However,

instrumentation is an important adjunct used by speech and language therapy teams to aid in assessment, record-keeping and some treatment. The development of high-quality and low-cost digital video cameras means that teams now have well-archived recorded speech assessments, which allow for audit and research to be carried out as well as to show progress in an individual's treatment.

The perceptual assessment of hypernasality has been found to correlate well with the 'nasalance' scores of the nasometer. The Kay Nasometer (Kay Elemetrics Corp., Lincoln Park, N.J., USA) is a microcomputer-based instrument which has microphones positioned on either side of a separating plate placed between mouth and nose. The acoustic signal from each microphone is detected and computed to give a ratio of nasal to 'nasal plus oral' energy, the nasalance score. Recording scores before and after secondary speech surgery can give an objective score of change. Norms have been established for some populations (ages and different accent populations) but by no means all and it is recommended that this work is undertaken to give more validity to results [18]. It should also never be seen as a substitute for the perceptual speech assessment.

Speech and Language Therapy Treatment

There are key times when the child might receive speech therapy. It should be remembered that there must be adequate velopharyngeal function for therapy to be wholly successful. Some teams advocate 'early intervention' during the second half of the first year of the child's life. This sometimes takes the form of workshops in which families are taught about early speech and language development and given suggestions for games designed to encourage the development of oral speech sounds. For direct speech work, the child needs to have the attention and listening skills and motivation to engage in speech sound work. The therapist needs skill in engaging the young

child and needs to tailor their style to the individual in order to facilitate and support change in their speech sound system. If the child or family find it difficult to engage in the therapy process at any age, this may have a negative impact on progress. Each case needs to be assessed individually and readiness for therapy is always an important skill to assess. Children may go through a period when therapy seems to be making little change. A break in treatment to allow the child to mature further followed by a later attempt may have more success. Quite startling progress may be made in the child's first year at school as learning in the classroom and peer relationships can have a considerable positive impact on a child. The older child with speech sound difficulties may find it difficult to change patterns which have been laid down over many years. However, the older child may be more motivated to change his or her speech.

Working Relationships within the Multidisciplinary Team

The importance of team working cannot be overemphasised. In most teams, a cleft specialist speech and language therapist will be present at multidisciplinary cleft clinics. It is not uncommon to hear the speech and language therapist, orthodontist, surgeon and psychologist working out with the patient how to schedule treatments not only in the most effective order but also at the best time for them. This cohesive approach is far easier to achieve when all relevant professionals are together in the clinic. The role of the speech and language therapist during the clinic visit is to ensure any speech concerns are assessed, recommendations given and where necessary, therapy or further investigations carried out. The speech and language therapist will also feedback to the surgeon on how the child is progressing as a result of the cleft surgery. Liaison with local speech and language therapy services, education and social

services is often needed as a result of clinic visits, particularly if the child has additional learning or health problems. There is regular liaison with all the different members of the multidisciplinary team but particular relationships are described below.

Clinical Nurse Specialist

The speech and language therapist works closely with the clinical nurse specialist for cleft. The nurse specialist has a close relationship with the family of the child, which has developed from the first days after diagnosis when they have provided much needed accurate information and support. As the baby becomes a toddler the focus of the child's development moves forward towards speech and language and in some teams a joint appointment at the age of 18 months to 2 years for the child and family with the nurse specialist and speech and language therapist is carried out. This helps with continuity of care for the family. These combined appointments serve to reduce the burden of care on the families for whom medical appointments may be numerous.

The Orthodontist

The orthodontist and speech and language therapist sometimes work together to treat patients with persisting speech difficulties. In spite of having received appropriate speech therapy, some children struggle to achieve clear anterior speech sounds such as [p], [b], [t], [d], [s], [z]. Their tongue positioning is retracted but they do not seem to be able to achieve the correct sound by imitation and conventional speech therapy alone. In a very few cases, electropalatography (EPG) may help part of the therapeutic process [23]. An EPG plate is a custom-made acrylic dental plate in which multiple sensors are embedded in the anterior section (fig. 2a). The child cannot wear the appliance if they are in an active orthodontic phase of treatment. The therapist and patient both wear their plate, which is connected to a desktop or portable computer. The tongue

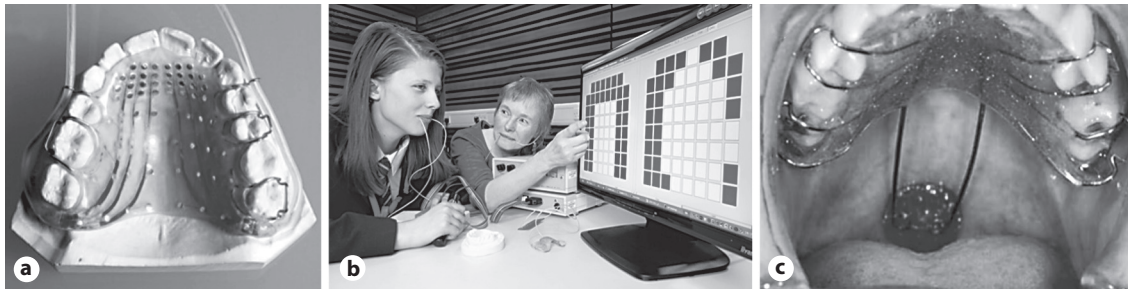


Fig. 2. **a** An EPG plate on a cast showing the position of the electrodes in the acrylic plate (copyright: Articulate Instrument Ltd). **b** Patient attempting to copy a therapist's speech pattern (photographer: Dougie Barnett; copyright: Queen Margaret University, Edinburgh, UK). **c** Speech bulb in situ (courtesy of Alex Cash).

contact illuminates the sensors and distinct sound patterns can be copied with immediate visual feedback provided on the computer screen. This shows the child's tongue contacts on the left-hand side of the screen and therapist's contacts on the right-hand side. The child can now try to copy the pattern which shows up in real time (fig. 2b). A recent Cochrane Review looking at the evidence for the effectiveness of this type of treatment concluded that, 'the current evidence supporting the efficacy of EPG is not strong and there remains a need for high-quality randomised controlled trials to be undertaken in this area' [24].

Palatal Lifts and Speech Bulbs

Another joint area of working with the orthodontist and surgeon involves some patients with VPD for whom surgery to overcome the problem is not possible or appropriate for a variety of reasons [25]. The orthodontist is able to make a dental plate with either a lift on the posterior portion or a speech bulb (fig. 2c). A lift is most likely to be helpful in a person with a probable neurological reason for a poorly moving soft palate. The lift is used during speaking to create a shelf to keep the palate raised thereby reducing

hypernasality or nasal emission. A speech bulb is used in cases where there is a shortage of tissue in the palate area sometimes caused by tumour removal.

Conclusions

The normal development of speech and speech problems that can arise in the cleft palate population have been discussed. Management and multidisciplinary approaches to treatment have been described. Effective communication in the form of spoken language is a large part of how others perceive us. The speech and language therapist aims to help give each child the best possible outcome for speech. Ideally the child should start formal education with as near-normal speech as possible. For some children though, there may be many years of therapy and several operations to help them achieve their best speech. The aim of all treatments for the individual born with cleft lip and/or palate is to allow the individual to achieve the best possible outcome in order to allow them to live a balanced and fulfilling life.

References

- 1 Clinical Standards Advisory Group: Cleft Lip and/or Palate. London, HMSO, 1998.
- 2 McWilliams BJ, Morris HL, Shelton RL: Cleft Palate Speech, ed 3. Philadelphia, Decker, 1990.
- 3 Grunwell P, Russell J: Vocalisations before and after cleft palate surgery – a pilot study. *Br J Disord Commun* 1987;22:1–17.
- 4 Henningsson G: Cleft palate babbling related to time of palate repair; in Kriens O (ed): What Is a Cleft Lip and Palate? Proceedings of an Advanced Workshop. New York, Thieme, 1987.
- 5 Spriestersbach DC, Dickson DR, Fraser FC, Horowitz SL, McWilliams BJ, Paradise J, Randall P: Clinical research in cleft lip and cleft palate: the state of the art. *Cleft Palate J* 1973;10:113–165.
- 6 Grunwell P, Russell J: Phonological development in children with cleft lip and palate. *Clin Linguist Phonet* 1988;2:75–95.
- 7 TOPS Trial Protocol. Version 2.1, 2010.
- 8 Gosain AK, Conley SF, Marks S, Larson DL: Submucous cleft palate: diagnostic methods and outcomes of surgical treatment. *Plast Reconstr Surg* 1996;97:1497–1509.
- 9 Paradise JL, Bluestone CD, Felder H: The universality of otitis media in 50 infants with cleft palate. *Paediatrics* 1969;44:35–42.
- 10 Shprintzen RJ, Siegel-Sadewitz VL, Amato J, Goldberg RB: Anomalies associated with cleft lip, cleft palate or both. *Am J Med Genet* 1985;4:585–595.
- 11 Lees M: Genetics of cleft lip and palate; in Watson AC, Sell DA, Grunwell P (eds): Management of Cleft Lip and Palate. London, Whurr, 2001, pp 95–96.
- 12 Stengelhofen J: The nature and causes of communication problems in cleft palate; in Stengelhofen J (ed): Cleft Palate: The Nature and Remediation of Communication Problems. London, Churchill Livingstone, 1989, pp 1–29.
- 13 Hall C, Golding-Kushner KJ: Long-term follow-up of 500 patients after palate repair performed prior to 18 months of age. 6th International Congress on Cleft Palate and Related Craniofacial Anomalies, Jerusalem, 1989.
- 14 Sell DA, Harding A, Grunwell P: A screening assessment of cleft palate speech: GOS.SPASS (Great Ormond Street Speech Assessment). *Eur J Dis Commun* 1994;29:1–15.
- 15 Sell DA, Harding A, Grunwell P: Revised GOS.SPASS (98): speech assessment for children with cleft palate and/or velopharyngeal dysfunction. *Int J Lang Commun* 1999;34:7–33.
- 16 Sell D, John A, Harding-Bell A, Sweeney T, Hegarty F, Freeman J: Cleft Audit Protocol for Speech (CAPS-A): a comprehensive training package for speech analysis. *Int J Lang Commun Disord* 2009;44:529–548.
- 17 Britton L: Auditing against standards for speech – closing the audit loop: 3 years' data. Craniofacial Society of Great Britain Annual Scientific Conference, 2011.
- 18 Sell D, Grunwell P: Speech assessment and therapy; in Watson AC, Sell DA, Grunwell P (eds): Management of Cleft Lip and Palate. London, Whurr, 2001, pp 74–75.
- 19 Albery E, Grunwell P: Consonant articulation in different types of cleft lip and palate; in Grunwell P (ed): Analysing Cleft Palate Speech. London, Whurr, pp 83–110.
- 20 Trost-Cardomone JE: Coming to terms with VPI: a response to Loney and Bloem. *Cleft Pal J* 1989;26:1.
- 21 Enderby P, Emerson J: Cleft palate; in Enderby P, Emerson J (eds): Speech and Language Therapy: Does It Work? Whurr, London, 1995, pp 142–165.
- 22 Sell D: Issues in perceptual speech analysis in cleft palate and related disorders: a review. *Int J Lang Commun Disord* 2005;40:103–121.
- 23 Hardcastle WJ, Gibbon F: Electro-palatography and its clinical applications; in Ball MJ, Code C (eds): Instrumental Clinical Phonetics. London, Whurr, 1987.
- 24 Lee AS-Y, Law J, Gibbon FE: Electro-palatography for articulation disorders associated with cleft palate. *Cochrane Database Syst Rev* 2009;3:CD006854.
- 25 Sell D, Mars M, Worrell E: Process and outcome study of multidisciplinary prosthetic treatment for velopharyngeal dysfunction. *Int J Lang Commun Disord* 2006;41:495–511.

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Future Directions: Molecular Approaches Provide Insights into Palatal Clefting and Repair

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Abstract

Normal development of the palate depends on spatial and temporal coordination of complex cellular processes and tissue-tissue interactions. Because these processes are quite sensitive to environmental and genetic perturbation, clefts of the palate are among the most common congenital anomalies seen in live births. The clinical burden of cleft palate is significant, as conventional treatments include surgical repair combined with long-term rehabilitation. Affected children may require multiple operations and often have secondary problems such as perturbed speech development, dental occlusion, maxillary growth deficiencies and otitis media. Recent reports, from patient studies and mouse models, have implicated a number of genes in palatogenesis. It is difficult to pinpoint the direct pathological effects of specific genes in humans; therefore, the majority of mechanistic insights have derived from murine models. Furthermore, recent technological advances have made mice an ideal system for studying the signalling events associated with cleft palate. This review discusses several illustrative examples of genetic or molecular studies in which in utero reversal of cleft palate reveals sequential requirements in palate formation. As we develop a more comprehensive understanding of the genetic mechanisms underlying normal and pathological palate development, we can begin to consider the possibility of molecular tools to complement or even replace surgical interventions.

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Cleft Palate

Cleft palate is a common birth defect, occurring in roughly 1 in 1,000 births [1]. Affected children often require surgical closure of the cleft within the first several years of life. These children then face continuing challenges, such as perturbed facial growth, speech impairment, dental occlusion and hearing defects. Many children require repeat surgeries and ongoing medical care, including therapy to establish normal speech patterns. In addition to speech, they have substantial dental problems that may require orthodontic intervention [1]. Not infrequently, these children also have associated midface hypoplasia due to palatal scarring on the growing maxilla. Typically, this can require complicated maxillofacial surgery to re-establish normal dental relationships and facial form. Finally, eustachian tube dysfunction frequently leads to fluid accumulation in the middle ear. This situation commonly entails placement of myringostomy tubes one or more times in the first 5 years of life, as fluid must be drained to prevent hearing loss. In summary, children with cleft palate require multiple operations to close the cleft in order to establish normal facial form, proper dental positioning, and to prevent hearing loss [1].

Development of the Secondary Palate

In recent years, a number of excellent studies have examined the development of the lip and palate as well as the complex aetiology of palatal clefts. In brief, the embryonic hard palate develops stereotypically [2]. The palatal shelves bud out from the oral sides of the maxillary prominences. These swellings initially grow vertically to flank the tongue, then rotate to a position above the tongue. There is then a second growth phase in which the palatal shelves extend medially, eventually meeting at the midline. Apposition of the opposing palatal shelves is followed by adherence at a midline epithelial seam (MES) and subsequent dissolution of the seam leads to palatal fusion. In addition, multiple signalling pathways, including Hedgehog (Hh), fibroblast growth factor, and transforming growth factor- β (TGF β) are important in coordinating the progression of palate development [3–5].

Clefts of the secondary palate can result from defects during any step in palatogenesis [6, 7]. For example, during the neural crest migration stages, tissue contributions to the maxillary prominence or changes in anterior-posterior or mediolateral patterning will affect initial segregation of the palatal primordia. Insufficient neural crest contributions will lead to maxillary hypoplasia and subsequent insufficiency. As the palatal shelf begins to grow, changes in proliferation, apoptosis or oriented growth can affect the size and placement of the shelves. These events are controlled in part by signalling between the mesenchymal and epithelial tissues in the palate. In addition, elevation of the palatal shelves can be blocked by steric hindrance from the tongue, which may fail to descend due to muscle defects or a small mandible. The extracellular matrix and forces intrinsic to the palate are also thought to play roles in palate rotation. Finally, fusion of the palate can go wrong if the shelves do not extend enough to meet, if the shelves do not adhere at the midline, or if there are defects in dissolution of the MES.

Thus, proper development of the palate requires the organization of multiple processes such as tissue morphogenesis, proliferation, differentiation and apoptosis.

Mouse Models Reveal Sequential Developmental Requirements

Development of the palate requires coordination of different tissues and signalling pathways, therefore this process must be assessed in animal models rather than in isolated cells. In the past few decades, the ease of genetic and chemical manipulation in murine models has made the mouse the premier system for studying palatogenesis. While the anatomical development of the mammalian secondary palate has been well established, less is understood about critical cellular and molecular mechanisms. This is due in large part to difficulties assessing direct molecular interactions. Even when genetic or environmental associations are found, it can be difficult to ascribe specific roles to individual factors. Often, the researcher evaluates the consequences of a molecular change by examining the responses of many affected tissues. These tissues signal to one another throughout development, making it difficult to discriminate between immediate cellular changes and later secondary effects. However, recent genetic approaches in mouse, combined with embryological and chemical manipulations, have shed new light on the signalling hierarchies regulating palatogenesis.

Genetic Rescue of Palatal Shelf Outgrowth Reveals Epithelial-Mesenchymal Networks

Growth of the palatal shelves is necessary for the palatal primordia to reach the appropriate size during the vertical extension phase. In addition, after rotation of the palatal shelves, growth also contributes to the expansion of the palatal shelves toward the midline. This growth is dictated by a

series of reciprocal epithelial-mesenchymal interactions, which have been revealed in a number of elegant mouse experiments.

In humans, mutations in the *MSX1* gene are associated with isolated cleft palate and tooth agenesis [8]. Knockouts of the gene in mouse had previously identified a role for *Msx1* in clefting [9], and analysis of the mouse mutants suggested that defects in cell proliferation affected palatal outgrowth, rather than fusion [10]. During the vertical growth stage, *Msx1* is known to be required for expression of bone morphogenetic proteins (BMPs) in the palatal mesenchyme, as well as *Sonic Hedgehog* (*Shh*) in the medial edge epithelium. Thus, Zhang et al. [10] hypothesized that a loss of BMP signalling in the mesenchyme might underlie the proliferation defect in *Msx1* mutant mice. They engineered transgenic mice expressing human BMP4 under the control of the mouse *Msx1* promoter. This transgene was able to rescue the cleft palate phenotype (as well as neonatal lethality) of *Msx1*^{-/-} mice. Furthermore, they saw a return of epithelial *Shh* expression and subsequent *Bmp2* expression in the mesenchyme. In a converse experiment, they tested the inductive abilities of *Shh* in the absence of *Msx1*. Application of *Shh*-soaked beads induced *Bmp2* induction without induction of *Bmp4*. Taken together, these experiments revealed a signalling network in which *Msx1* is required for induction of *Bmp4* but not *Shh* and *Bmp2*.

These data suggested that mesenchymal cell proliferation could be stimulated by *Shh* expression in the epithelia, downstream of *Bmp4* and upstream of *Bmp2*. Han et al. [11] recently tested the possibility that increased epithelial *Shh* would be sufficient to rescue mesenchymal proliferation defects in *Msx1* mutant palates. To do this, they made use of *Dlx5* mutants, which have aberrant oro-nasal patterning of the palatal shelf. In mice lacking *Dlx5* the oral region of the palatal shelf is expanded. This increase appears to be due to an increase in the *Shh* domain in the epithelium, which then signals to the underlying mesenchyme,

inducing excessive proliferation. Han et al. [11] then generated mouse mutants lacking both *Msx1* and *Dlx5* and found that normal palate development was restored in these animals. Furthermore, in order to pinpoint the role for *Dlx5* control of *Shh* expression, they treated explanted *Msx1*^{-/-}; *Dlx5*^{-/-} palates with an antibody that blocks Hh signalling. In these treatments, mesenchymal cell proliferation was reduced, consistent with a role for increased *Shh* signalling being directly responsible for the proliferation-mediated rescue.

Taken together, these approaches provide important insights into the genetic control of outgrowth of the palate. With the increased availability of mouse mutants and transgenics, systematic genetic or pharmacological rescue of palatal outgrowth defects will help establish the molecular networks controlling this process.

Reversal of Palatal Elevation Defects Uncovers Neural Requirements

Shelf elevation requires depression of the tongue as well as a 90° rotation of the palatal shelves, from a vertical, downward pointing direction to a horizontal position. Movement of the tongue is also important; if the tongue does not depress, it can hinder rotation of the shelf. There are a number of proposed mechanisms for coordination of palatal shelf elevation including fetal movements and muscular contractions. In human fetuses, mouth opening and tongue withdrawal is observed during gestation at about weeks 9–10, when elevation of the palate is taking place [12]. In addition, forces generated by cellular reorganisation or the extracellular matrix may play critical roles.

For some years, researchers studying γ -aminobutyric acid (GABA) signalling have been puzzled by palatal phenotypes in mutant mice [13]. GABA is a neurotransmitter and signalling molecule required in multiple phases of neurogenesis, ranging from neuronal production and migration to differentiation and synaptic

integration. However, mutating a number of GABA pathway genes in mice results in cleft palate. These include genes required for GABA synthesis (*Gad1*, also called *Gad67*), GABA transport (*Viaat*) and GABA reception (*Gabrb3*) [14–17]. Furthermore, GABA pathway genes are weakly associated with non-syndromic cleft lip and palate in humans [16, 18, 19]. Most of these genes do not appear to be expressed in the oral cavity. In addition, specific deletion of *Gad1* in the neural tissues also results in cleft palate. Taken together, these data point toward CNS or functional muscle requirements for GABA signalling during development of the palate. However, several groups had previously reported that conditionally deleting the downstream receptor, *Gabrb3*, using neuronal-specific drivers did not result in palatal defects [16, 20], which would imply that requirements for GABA reception are intrinsic to the oral tissues.

To determine the aetiology of cleft palate in *Gad1*^{-/-} animals, Tsunekawa et al. [21] first examined fetal movements in normal mice by ultrasound imaging. They observed regular mouth movements and tongue withdrawal by E14, when palatal shelves are preparing to rotate. In comparison, mutant mice did not move as much. In a subsequent study, Iseki et al. [22] used in utero surgical approaches to remove the tip of the tongue. Surprisingly, removal of the tongue was sufficient to allow palatal shelf rotation within 30 min. This result implies that fetal movements are necessary for displacement of the tongue; when the tongue is removed, forces intrinsic to the palatal shelves lead to rapid rotation of the shelves.

To test the elevation abilities of GABA-deficient palatal shelves, Oh et al. [23] used an explant culture system, allowing them to remove the tongue and track subsequent steps of palatal development. They showed that palatal shelves from both *Viaat* and *Gad1* mutant mice were able to undergo normal shelf formation, growth, elevation and fusion, consistent with an in vivo role for fetal tongue movements.

Loss of the GABA_A receptor *Gabrb3*, mentioned above, also leads to cleft palate; therefore, Oh et al. [23] reasoned that pharmacologically activating the GABA receptor might rescue the phenotypes of the *Viaat* and *Gad1* mutant mice. Indeed, injection of the GABA agonist muscimol into pregnant dams between E13.5 and E16.0 rescued shelf elevation in 50% of the mutants treated. Because GABA receptors do not appear to be expressed in the mouse palate, muscimol is more likely to be binding to GABA receptors in the nervous system, thereby regulating fetal brain activity.

Hierarchical Signalling Requirements during Palatal Fusion

Once the bilateral palatal shelves have elevated, they grow toward the midline and approximate to form the MES. The MES must disappear for palatal fusion to occur, however the underlying mechanisms are still controversial. MES dissolution has been hypothesized to require apoptosis, epithelial-mesenchymal transformation and cell migration. The evidence for epithelial-mesenchymal transformation and cell migration are confusing and contradictory [reviewed in 6], however the evidence for apoptosis of MES cells is quite compelling.

A number of cell signalling factors have been implicated in regulation of palatal fusion, including TGF β , platelet-derived growth factor, epidermal growth factor and ephrin signalling [2, 6]. In general, these mouse mutants were identified based on the persistence of the MES in cultured explants, which can be used to bypass earlier outgrowth effects. Explants can then be tested for sensitivity to exogenous growth factors or chemical regulators. Thus, the existence of mouse mutants combined with embryological culturing methods allows us to examine the genetic hierarchy controlling dissolution of the palatal seam.

Molecularly, transforming growth factor- β 3 (TGF β 3) signalling appears to be pivotal in

controlling the MES. Mutant mice lacking TGF β 3 have isolated cleft palate due to a failure of palatal fusion [24]. TGF β 3 is primarily expressed in the MEE prior to dissolution and appears to be required for both adherence of the opposing palatal shelves and subsequent induction of apoptosis in the MES [25]. Examination of downstream mutations in the TGF β pathway, including TGF β RII and Alk5 (TGF β RI), demonstrated that TGF β RII and Alk5 are also required specifically in the epithelium: in mutants, the MES overproliferates and does not undergo apoptosis, resulting in a persistent midline seam [26, 27]. Furthermore, overexpression of the downstream transcriptional effector Smad2 rescued the cleft palate in regions where the palatal shelves have successfully adhered [26].

The knowledge that TGF β 3 intimately controls dissolution of the MES then allows researchers to examine the ability of other candidate ligands to induce this activity and to establish an order of events. For example, in mouse mutants lacking the transcription factors *Snail1* and *Snail2* the MES does not form and palatal shelves fail to fuse [28]. TGF β 3 expression appears normal in these mice, suggesting that the *Snail* genes are acting downstream to TGF β 3 or in a parallel fusion pathway.

Furthermore, genetic methods can be used to address functional redundancy of the TGF β ligands: Yang and Kaartinen [29] tried to genetically rescue TGF β 3 mutants by expressing Tgfb1 in its place. These mice displayed only partial rescue of the palatal clefting, suggesting that TGF β ligands have differential activities.

A more difficult problem arises when genes are required during sequential stages or in multiple tissues during developmental progressions. For example, ephrins and their receptors (Ephs) are known to be important in adherence and midline fusion of many embryonic organs. Mutations in a number of ephrin pathway genes such as *Ephb2* and *Ephb3* do result in cleft palate [30]; however, they have an early defect in palatal shelf

outgrowth which prevents examination of later steps in palatogenesis. To complicate matters, Eph/ephrin binding leads to responses in both the Eph and ephrin-expressing cells, resulting in bidirectional signalling [reviewed in 31]. In the case of ephrinB1, it appears that forward signalling is important during shelf formation, while reverse signalling is required in the mesenchyme during shelf elevation [32]. Furthermore, Risley et al. [33] demonstrated that forward signalling by EphB2 and EphB3 are not required for palatal fusion.

Nevertheless, Eph/ephrin molecules are expressed in the MES. Studies in chicken suggest that Eph/ephrin reverse signalling can induce fusion in the absence of TGF β 3 and, furthermore, is required for TGF β 3-mediated palatal fusion [34]. These experiments merit further examination in mouse mutants, ideally with a tissue-specific rescue of TGF β 3 phenotypes, as with the Smad2 experiments mentioned above.

In many instances, development studies are hampered by a number of challenges. The existing mutation may have an early phenotype that obscures the later roles of a gene. This can be partially bypassed using conditional mutants and explant analyses. Genetic crosses can be performed, however mutational analysis and mouse breeding are laborious and expensive. Furthermore, comparison of mouse mutants can be difficult, as mutants are often bred in different genetic backgrounds and phenotypes can be variable.

Engineering Drug-Dependent Genetic Mutants

Studying the functions of specific proteins within an intact animal presents several challenges. Genetic deletion completely eliminates a protein, but since most proteins have roles in different tissues or at different stages of development, a knockout mouse may not survive to the desired stage of maturity. Pharmacologic approaches are attractive alternatives because small molecules

can be used to inhibit protein function in a genetically normal animal. These drugs can be administered and removed at specific times and are often reversible, providing attractive lead compounds for drug development.

In mammals, there exist two closely related forms of GSK-3 (α and β), both of which act in signalling pathways such as Wnt, insulin, TGF β and Hh signalling [reviewed in 35]. Because GSK-3 plays a pivotal role in multiple biological processes such as diabetes, Alzheimer's disease, neurodegeneration and cancer, many pharmaceutical companies are interested in targeting these proteins. However, the two isoforms of GSK-3 are structurally very similar; therefore, most of the engineered drugs cannot distinguish between the two proteins. This also makes it difficult to examine individual gene functions *in vivo*.

To study the function of signalling proteins in development our laboratory is combining the advantages of gene targeting with small molecule sensitivity. We are using an approach called inducible stabilization, wherein a non-toxic drug regulates the stability of any protein of interest. In this example, we use an 89-amino-acid tag, (FRB*), which can be fused to target genes. Fusion proteins are rapidly destabilized [36]. In the presence of a chemical ligand (rapamycin or rapamycin analogues), FRB*-tagged proteins dimerize with endogenous FKBP. This interaction stabilizes the fusion protein, restoring protein levels and activity. Using genetically engineered mice, we recently identified a sensitive period during which the kinase GSK-3 β is required during palatal development [37]. GSK3 β null mutant mice normally develop cleft palate. We set out to rescue the palatal defects by inducibly stabilizing the protein during 1- to 2-day windows in embryogenesis. We found that restoring the protein, *in utero*, between E13.5 and E15.0 was sufficient to partially or completely rescue the clefting. Our approach allowed us to identify the critical time window when GSK-3 β is needed, and to implicate GSK-3 β (rather than

GSK-3 α) in the process. Furthermore, the timing of the experiment suggests that GSK-3 β is important for horizontal growth, as maximum accumulation of GSK-3 β protein would occur by about E14+ to E15+. Consistent with this, mutant shelves do undergo rotation *in vivo* but do not meet at the midline.

In a separate study, He et al. [38] chose to examine tissue-specific requirements for GSK-3 β during palatogenesis. They generated conditional GSK-3 β mutants and found that deletion of the gene in the epithelium resulted in cleft palate. In their system, the palatal shelves were unable to elevate in culture; therefore, they speculate that GSK-3 β is controlling factors in the epithelia that are required for shelf rotation. These results, though inconsistent with our data, are intriguing. Differences may be due to genetic background, or other unknown factors [37, 38]. Regardless, in future studies, it will be very useful to combine traditional conditional approaches with new chemical approaches. For example, GSK-3 β FRB* could be expressed using tissue-specific promoters. This would allow conditional genetic loss of GSK-3 followed by chemically induced restoration of protein in a tissue-specific manner. Furthermore, cell and explant cultures are readily accessible to pharmacological approaches, and cellular responses are likely to be much faster than *in vivo* drug administration. Explants would also bypass maternal drug metabolism, which can further complicate *in vivo* studies.

In vivo Rescue of Cleft Palate

The consequences of a cleft palate are a burden not only to the affected patient, but also to their family. Early diagnosis can allow clinicians to anticipate postnatal needs as well as to educate and prepare the parents. *In utero* detection is becoming straightforward, raising the possibility that doctors may be able to reverse a cleft prior to birth. However, this will require careful regulation of

the amount of signalling factors involved in palate development.

At present, in utero repair of cleft palate seems unlikely. The signalling molecules implicated in palatal development are all potent, pleiotropic factors that are likely to affect other developmental processes. However, as we develop a better understanding of the molecular aetiology of cleft palate,

we can begin to consider manipulating genes, proteins and signalling pathways as therapeutic agents. Even a decrease in the number of repeat surgical procedures will be an improvement. Our hope is that in the future, more refined methods for regulating signalling may be combined with surgical approaches to improve growth, morphogenesis and healing.

References

- 1 Robin NH, Baty H, Franklin J, Guyton FC, Mann J, Woolley AL, Waite PD, Grant J: The multidisciplinary evaluation and management of cleft lip and palate. *South Med J* 2006;99:1111–1120.
- 2 Gritli-Linde A: Molecular control of secondary palate development. *Dev Biol* 2007;301:309–326.
- 3 Iwata J, Parada C, Chai Y: The mechanism of TGF- β signaling during palate development. *Oral Dis* 2011;17:733–744.
- 4 Rice R, Spencer-Dene B, Connor EC, Gritli-Linde A, McMahon AP, Dickson C, Thesleff I, Rice DP: Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. *J Clin Invest* 2004;113:1692–1700.
- 5 Cobourne MT, Xavier GM, Depew M, Hagan L, Sealby J, Webster Z, Sharpe PT: Sonic hedgehog signalling inhibits palatogenesis and arrests tooth development in a mouse model of the nevroid basal cell carcinoma syndrome. *Dev Biol* 2009;331:38–49.
- 6 Gritli-Linde A: The etiopathogenesis of cleft lip and cleft palate: usefulness and caveats of mouse models. *Curr Top Dev Biol* 2008;84:37–138.
- 7 Ferguson MW: Palate development. *Development* 1988;103(suppl):41–60.
- 8 Van den Boogaard MJ, Dorland M, Beemer FA, van Amstel HK: MSX1 mutation is associated with orofacial clefting and tooth agenesis in humans. *Nat Genet* 2000;24:342–343.
- 9 Satokata I, Maas R: Msx1-deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nat Genet* 1994;6:348–356.
- 10 Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen Y: Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. *Development* 2002;129:4135–4146.
- 11 Han J, Mayo J, Xu X, Li J, Bringas P Jr, Maas RL, Rubenstein JL, Chai Y: Indirect modulation of Shh signaling by Dlx5 affects the oral-nasal patterning of palate and rescues cleft palate in Msx1-null mice. *Development* 2009;136:4225–4233.
- 12 Humphrey T: The relation between human fetal mouth opening reflexes and closure of the palate. *Am J Anat* 1969;125:317–344.
- 13 Condie BG, Bain G, Gottlieb DI, Capecchi MR: Cleft palate in mice with a targeted mutation in the γ -aminobutyric acid-producing enzyme glutamic acid decarboxylase 67. *Proc Natl Acad Sci USA* 1997;94:11451–11455.
- 14 Wee EL, Zimmerman EF: GABA uptake in embryonic palate mesenchymal cells of two mouse strains. *Neurochem Res* 1985;10:1673–1688.
- 15 Wee EL, Norman EJ, Zimmerman EF: Presence of γ -aminobutyric acid in embryonic palates of AJ and SWV mouse strains. *J Craniofac Genet Dev Biol* 1986;6:53–61.
- 16 Hagiwara N, Katarova Z, Siracusa LD, Brilliant MH: Nonneuronal expression of the GABA β subunit gene is required for normal palate development in mice. *Dev Biol* 2003;254:93–101.
- 17 Asada H, Kawamura Y, Maruyama K, Kume H, Ding RG, Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K: Cleft palate and decreased brain γ -aminobutyric acid in mice lacking the 67-kDa isoform of glutamic acid decarboxylase. *Proc Natl Acad Sci USA* 1997;94:6496–6499.
- 18 Vieira AR, Howe A, Murray JC: Studies of γ -aminobutyric acid type A receptor β 3 (GABRB3) and glutamic acid decarboxylase 67 (GAD67) with oral clefts. *Am J Med Genet A* 2008;146A:2828–2830.
- 19 Kanno K, Suzuki Y, Yamada A, Aoki Y, Kure S, Matsubara Y: Association between nonsyndromic cleft lip with or without cleft palate and the glutamic acid decarboxylase 67 gene in the Japanese population. *Am J Med Genet A* 2004;127A:11–16.
- 20 Ferguson C, Hardy SL, Werner DF, Hileman SM, Delorey TM, Homanics GE: New insight into the role of the β 3 subunit of the GABAA-R in development, behavior, body weight regulation, and anesthesia revealed by conditional gene knockout. *BMC Neurosci* 2007;8:85.
- 21 Tsunekawa N, Arata A, Obata K: Development of spontaneous mouth/tongue movement and related neural activity, and their repression in fetal mice lacking glutamate decarboxylase 67. *Eur J Neurosci* 2005;21:173–178.

- 22 Iseki S, Ishii-Suzuki M, Tsunekawa N, Yamada Y, Eto K, Obata K: Experimental induction of palate shelf elevation in glutamate decarboxylase 67-deficient mice with cleft palate due to vertically oriented palatal shelf. *Birth Defects Res A Clin Mol Teratol* 2007;79:688–695.
- 23 Oh WJ, Westmoreland JJ, Summers R, Condie BG: Cleft palate is caused by CNS dysfunction in *Gad1* and *Viaat* knockout mice. *PLoS One* 2011;5:e9758.
- 24 Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, Groffen J: Abnormal lung development and cleft palate in mice lacking TGF- β 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet* 1995;11:415–421.
- 25 Dudas M, Nagy A, Laping NJ, Moustakas A, Kaartinen V: Tgf- β 3-induced palatal fusion is mediated by Alk-5/Smad pathway. *Dev Biol* 2004;266:96–108.
- 26 Xu X, Han J, Ito Y, Bringas P Jr, Urata MM, Chai Y: Cell autonomous requirement for *Tgfb2* in the disappearance of medial edge epithelium during palatal fusion. *Dev Biol* 2006;297:238–248.
- 27 Dudas M, Kim J, Li WY, Nagy A, Larsson J, Karlsson S, Chai Y, Kaartinen V: Epithelial and ectomesenchymal role of the type I TGF- β receptor ALK5 during facial morphogenesis and palatal fusion. *Dev Biol* 2006;296:298–314.
- 28 Murray SA, Oram KF, Gridley T: Multiple functions of Snail family genes during palate development in mice. *Development* 2007;134:1789–1797.
- 29 Yang LT, Kaartinen V: *Tgfb1* expressed in the *Tgfb3* locus partially rescues the cleft palate phenotype of *Tgfb3* null mutants. *Dev Biol* 2007;312:384–395.
- 30 Orioli D, Henkemeyer M, Lemke G, Klein R, Pawson T: *Sek4* and *Nuk* receptors cooperate in guidance of commissural axons and in palate formation. *Embo J* 1996;15:6035–6049.
- 31 Pasquale EB: Eph-ephrin bidirectional signaling in physiology and disease. *Cell* 2008;133:38–52.
- 32 Davy A, Aubin J, Soriano P: Ephrin-B1 forward and reverse signaling are required during mouse development. *Genes Dev* 2004;18:572–583.
- 33 Risley M, Garrod D, Henkemeyer M, McLean W: EphB2 and EphB3 forward signalling are required for palate development. *Mech Dev* 2009;126:230–239.
- 34 San Miguel S, Serrano MJ, Sachar A, Henkemeyer M, Svoboda KK, Benson MD: Ephrin reverse signaling controls palate fusion via a PI3 kinase-dependent mechanism. *Dev Dyn* 2011;240:357–364.
- 35 Frame S, Cohen P: GSK3 takes centre stage more than 20 years after its discovery. *Biochem J* 2001;359:1–16.
- 36 Stankunas K, Bayle JH, Gestwicki JE, Lin YM, Wandless TJ, Crabtree GR: Conditional protein alleles using knockin mice and a chemical inducer of dimerization. *Mol Cell* 2003;12:1615–1624.
- 37 Liu KJ, Arron JR, Stankunas K, Crabtree GR, Longaker MT: Chemical rescue of cleft palate and midline defects in conditional GSK-3 β mice. *Nature* 2007;446:79–82.
- 38 He F, Popkie AP, Xiong W, Li L, Wang Y, Phiel CJ, Chen Y: Gsk3 β is required in the epithelium for palatal elevation in mice. *Dev Dyn* 2011;239:3235–3246.

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